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THE UNIVERSITY OF ALBERTA  
STUDIES ON THE MYOSIN NUCLEOSIDE TRIPHOSPHATASE  
AND THE ACTIN-MYOSIN INTERACTION UNDER THE INFLUENCE OF  
CALCIUM AND MAGNESIUM AT LOW CONCENTRATIONS OF POTASSIUM CHLORIDE

by



EDWARD ARTHUR SUGDEN

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies for acceptance, a thesis entitled "Studies on the Myosin Nucleoside Triphosphatase and the Actin-Myosin Interaction under the Influence of Calcium and Magnesium at Low Concentrations of Potassium Chloride" submitted by Edward Arthur Sugden in partial fulfilment of the requirements for the degree of Doctor of Philosophy.





## ABSTRACT

Muscular contraction involves the enzymatic hydrolysis of nucleoside triphosphates by myosin and actomyosin in the presence of the divalent cations  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$ . The chemical energy thus released is converted into the mechanical energy of contraction. In the present study, the enzymatic activity of myosin and actomyosin and the superprecipitation process have been investigated with the objective of clarifying the roles of the divalent cations and the nature of the actin-myosin interaction in the contractile process.

In the presence of  $\text{Ca}^{2+}$ , the rate of ATP or ITP hydrolysis by myosin or actomyosin suggests that a relatively weak binding of  $\text{Ca}^{2+}$  either with the protein or with the substrate molecule is responsible for the activation of the enzymatic activity, or the production of superprecipitated actomyosin particles. According to the experiments with an ultrafiltration technique, the binding of  $\text{Ca}^{2+}$  to myosin proceeds in at least two steps, the first occurring at one site on every 500,000 daltons of myosin with an apparent association constant,  $K_{app}$ , of  $1.3 \times 10^6 \text{ M}^{-1}$ . However, the second is so weak that its binding parameters cannot be determined by the method used. The first type of  $\text{Ca}^{2+}$  binding is not observed with N-ethylmaleimide-modified myosin yet this modified myosin shows the  $\text{Ca}^{2+}$  activation of the adenosine triphosphatase and inosine triphosphatase.

The  $\text{Mg}^{2+}$  effects on myosin or actomyosin enzymatic activity can be related to a binding reaction of  $\text{Mg}^{2+}$  to myosin having a



Kapp of  $\sim 10^6 \text{ M}^{-1}$ . A binding of  $\text{Mg}^{2+}$  occurs at the site in myosin where the  $\text{Ca}^{2+}$  binds tightly. The value of Kapp for the  $\text{Mg}^{2+}$  binding calculated by assuming a competition between  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$  for the same site is  $2.1 \times 10^5 - 3.0 \times 10^5 \text{ M}^{-1}$ . A direct measurement of  $\text{Mg}^{2+}$  binding to myosin shows a Kapp value of  $\sim 10^6 - 10^7 \text{ M}^{-1}$ . The behaviour of the enzymatic activity of myosin on ATP or ITP in the presence of both  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$  is consistent with the explanation that the  $\text{Mg}^{2+}$  inhibition is due to the tight binding of  $\text{Mg}^{2+}$  to myosin, whereas the  $\text{Ca}^{2+}$  activation is caused by a weak binding of  $\text{Ca}^{2+}$  to myosin or by  $\text{CaNTP}^{2-}$  or by both.

The binding reaction of actin with myosin and its subfragment was studied under the influence of  $\text{Mg}^{2+}$  and ATP. One actin monomer binds to one subfragment molecule and also to one molecule of myosin. It is explained that if only one of the two sites in myosin react, a second binding would be sterically hindered by the presence of bound actin. Actin binds to myosin with a Kapp of  $3.2 \times 10^6 - 5.0 \times 10^6 \text{ M}^{-1}$  while the maximum Kapp of actin-subfragment is  $2.0 \times 10^4 \text{ M}^{-1}$ . The binding experiments and the light scattering analysis on mixtures of actin and myosin suggest that the light meromyosin portion of myosin is necessary for stabilizing the actin-myosin interaction and the superprecipitation of actomyosin.





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## LIST OF ABBREVIATIONS

AMP	adenosine 5'-monophosphate
ADP	adenosine 5'-diphosphate
ATP	adenosine 5'-triphosphate
DEAE-cellulose	diethylaminoethyl-cellulose
D.T.T.	dithiothreitol
EDTA	ethylenediaminetetraacetic acid
EGTA	ethyleneglycolbis ( $\beta$ -aminoethylether) N,N'- tetraacetic acid
F-actin	fibrous action (polymer of G-actin monomer units)
G-actin	globular actin
HMM	heavy meromyosin
HMM-S1	heavy meromyosin subfragment I (subfragment)
HMM-SII	heavy meromyosin subfragment II
IMP	inosine 5'-monophosphate
ITP	inosine 5'-triphosphate
K <sub>app</sub>	apparent association constant
LMM	light meromyosin
M <sub>w</sub>	weight average molecular weight
NEM	N-ethylmaleimide
NDP	nucleoside 5'-diphosphate
NTP	nucleoside 5'-triphosphate
pMB	para-mercuribenzoate
Pi	inorganic phosphate
PP	pyrophosphate



## LIST OF ABBREVIATIONS (continued)

$S_{20}^0, w$	intrinsic sedimentation coefficient in water at $20^{\circ}$ (in Svedberg units, S)
TCA	trichloroacetic acid
Tris-Cl	tris (hydroxymethyl) amino-methane -- chloride
$\mu$	ionic strength



## I. INTRODUCTION

The investigations of the Russian workers (Ljubimova and Engelhardt, 1939; Engelhardt et al., 1941) have led researchers to an understanding that the mechano-chemical energy conversion in muscle is founded on the interaction between actin and myosin. Myosin possesses the enzymatic property of hydrolyzing a nucleoside triphosphate (NTP) into a nucleoside diphosphate (NDP) and inorganic phosphate:



Much of the earlier evidence that this nucleoside triphosphatase (NTPase) activity of myosin is directly related to contractility has come from the experiments with actomyosin, a complex of actin and myosin, obtained as threads by extrusion of its solution in 0.5-0.6M KCl through an orifice into a dilute salt solution (Weber, 1958). Recently, Infante et al., (1965) demonstrated that in isolated living muscle fibers, ATP splitting is proportional to the tension developed. Although the polymerization of monomer actin (G-actin), which forms fibrous polymer (F-actin), is accompanied by hydrolysis of ATP attached to G-actin, this process seems too slow to contribute significantly to the consumption of ATP during muscle contraction (Hayashi, 1967).

At the time of the discovery of ATPase activity associated to the contractile protein, it was noted that this enzymatic action is affected strongly by certain divalent cations (Bailey, 1954). It is now known that two prominent divalent cations in muscle,  $\text{Ca}^{2+}$





and  $Mg^{2+}$ , activate actomyosin ATPase. For the ATPase action of myosin,  $Ca^{2+}$  remains as an activator, whereas  $Mg^{2+}$  becomes an inhibitor. Both  $Ca^{2+}$  and  $Mg^{2+}$  are also known to be essential for the contraction of living muscle. In the experiments with actomyosin threads, Weber and co-workers found that only  $Mg^{2+}$  is necessary for this model system to contract. This prompted Weber (1958) to suggest that  $Ca^{2+}$  acts as a deactivator of the "relaxing factor" which inhibits the contractile process unless  $Ca^{2+}$  reacts with this factor. In fact, this factor was found (Ebashi and Endo, 1968) to be the proteins tropomyosin and troponin rather than a granular component as Weber had previously thought.

It thus becomes feasible to consider the basic contractile system as being constructed by actin, myosin,  $Mg^{2+}$ , and ATP. In this system, the process of ATP hydrolysis catalysed by myosin is coupled to a tension developing step, and the role of  $Mg^{2+}$  is apparently in this coupling mechanism. It appears that detailed knowledge of the effects of  $Mg^{2+}$  on the system of actin, myosin and ATP helps in understanding the mechanism of energy conversion in muscle contraction.

One of the major problems in studying the actomyosin system has been that both actin (hereafter this term refers to F-actin unless specified otherwise) and myosin are large molecules and are at a state of a hydrated gel, except at high ionic strength, which prevents the application of various physical methods developed for studies of soluble proteins. A frequently used approach to the problem has been to compare the ATPase activity and the macroscopic physical properties such as turbidity and viscosity, of actomyosin suspensions. The



actomyosin gel shows an intensive syneresis, superprecipitation, when it is placed under the conditions which allow muscle fibers to contract, and tends to dissociate if the conditions are favourable for relaxation.

In a previous study of actomyosin suspensions, it was suggested that two types of binding reactions involving  $Mg^{2+}$  influence the reactions in a system of actomyosin and ATP: the first is a strong binding of  $Mg^{2+}$  to the protein, which accelerates the ATPase activity, and the second is a relatively weak binding to either the protein or ATP or both, which coincides with the increase in the rate of superprecipitation. Considering the fact that both  $Ca^{2+}$  and  $Mg^{2+}$  activate actomyosin ATPase it seemed of value to see if  $Ca^{2+}$  can replace  $Mg^{2+}$  in the process of superprecipitation. The results of such an experiment could indicate the specificity of  $Mg^{2+}$  in its effects on the actin-myosin interaction.

In the first stage of this study, the effects of  $Ca^{2+}$  and  $Mg^{2+}$  on myosin ATPase and ITPase were analysed to answer the following questions: do these cations exert their effects through modifying myosin or the substrate or both, and is there any qualitative difference between two cations in their influences on the enzymatic action? Secondly, an attempt was made to assess the efficiency of  $Ca^{2+}$ , if it can replace  $Mg^{2+}$ , in the reaction of actomyosin leading to superprecipitation. Thirdly, on the purpose of gaining an insight into the mode of interaction between actin and myosin, the  $Mg$ -activated ATPase activity was analysed on the assumption that it represents the amount of actomyosin formed. The results were compared with the data obtained

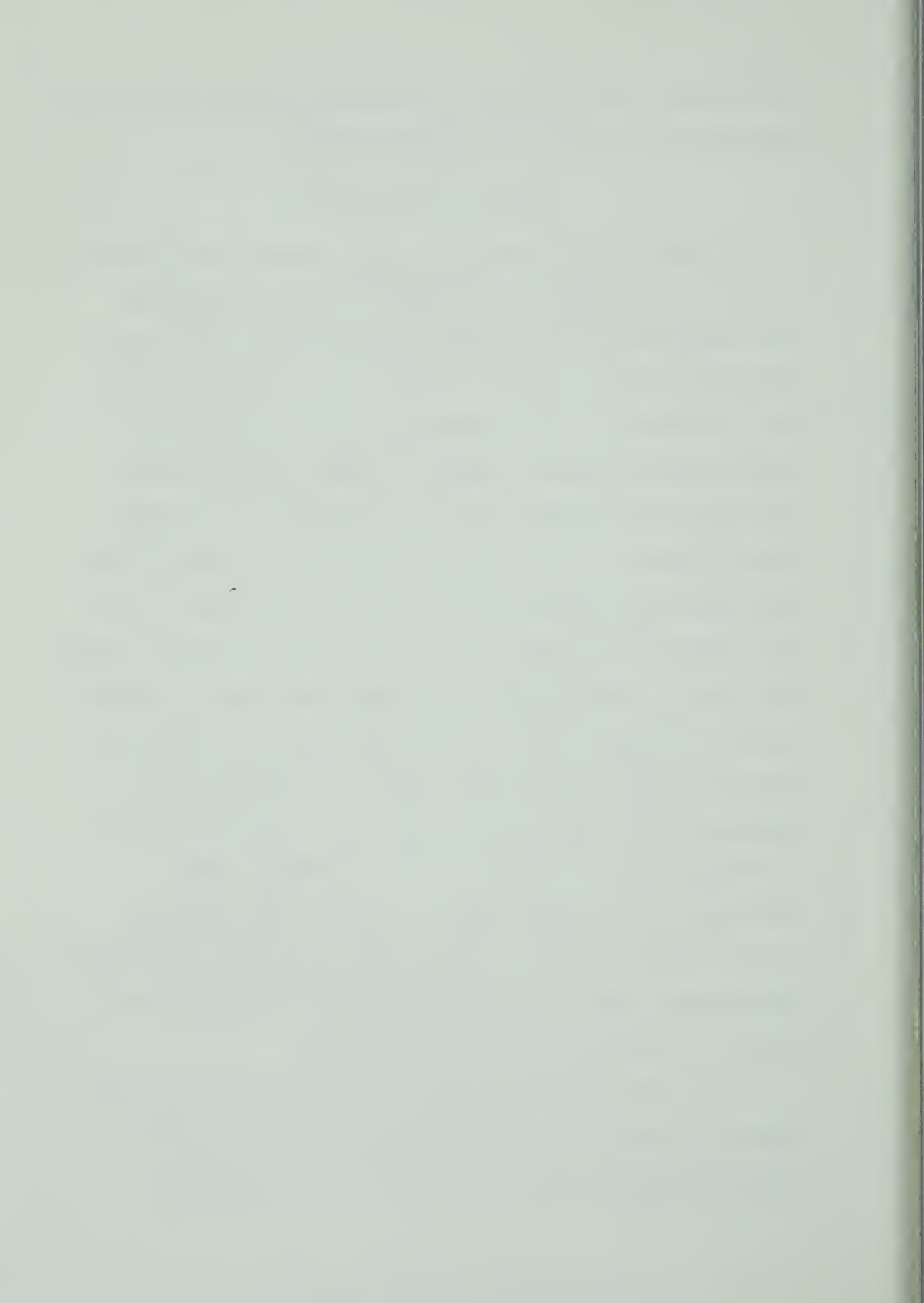


from the experiments in which the enzymatically active subfragment of myosin was used in place of the parent molecule.

#### Review of Previous Work

In regard to the activation of myosin adenosine triphosphatase by  $\text{Ca}^{2+}$ , it was proposed that the  $\text{CaATP}^{2-}$  complex is hydrolysed more rapidly than are other species of ATP (Nanninga, 1957; Blum and Felauer, 1959; Lowenstein, 1960; Tetas and Lowenstein, 1963; Luchi and Kritcher, 1967). According to the observations with nuclear magnetic resonance technique, divalent cations interact mainly with the triphosphate chain of ATP (Hammes et al., 1961; Cohn and Hughes, 1962), which may explain that the lability of the pyrophosphate bond depends on the nature of bound cation. It was also suggested by the same authors (Nanninga, 1959; Blum and Felauer, 1959) that the cations are bound to myosin, modifying its enzymatic activity. Recently, Taylor et al., (1970) analysed in detail the pre-steady state of myosin ATPase action to show that the dissociation of the enzyme-product complex is the rate limiting step of ATP hydrolysis: the dissociation rate of  $\text{MgADP}^-$ -enzyme is slower than that of  $\text{CaADP}^-$ -enzyme or free ADP bound with myosin. In this connection, Lowey and Luck (1969) reported that myosin binds with ADP more tightly in the presence of  $\text{Mg}^{2+}$  than in the presence of  $\text{Ca}^{2+}$ , or in the absence of divalent cations.

It was first observed by Singer and Barron (1944) that myosin ATPase is inhibited by a variety of sulfhydryl reagents. Later Kielly and Bradley (1956) showed that a titration of 10-12 of





the 33-36 sulfhydryl groups in one mole of myosin (500,000 daltons) with p-mercuribenzoate (pMB) leads to an activation of the myosin ATPase (see Table 1), whereas further titration results in a progressive inhibition. Sekine et al., in 1962 demonstrated that upon the activation of ATPase by N-ethylmaleimide (NEM), there are primarily two sulfhydryls per myosin molecule which are alkylated. Morales and his colleagues (Morales and Hotta, 1960; Rainford et al., 1964; Warren et al., 1966) interpreted this effect as being due to a local conformational change in the myosin molecule. The modified ATPase is clearly altered in its response to cations and it has been shown by Kitagawa et al., (1961) that pMB-modified myosin loses its tightly bound  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$ . In this regard, myosin which has been treated with pMB or NEM such that its ATPase is maximal, becomes a valuable tool for exploring the qualitative differences between  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$  effects on NTP hydrolysis.

It should be noted that there have been reports of differences in the ATPase activity of myosin prepared from red and white skeletal muscle (Sreter et al., 1966; Margreth et al., 1969). It was found that the activity of red muscle myosin is lower, responds more readily to changes in temperature or pH, and requires a higher amount of pMB to show the sulfhydryl effect than white muscle myosin. These observations suggest that there are various species of myosin molecule between muscle types.

The morphological structure of myosin is depicted as a two chain rod, at one end of which there is a part, called the head, where the sites for actin-binding and ATPase are situated (Fig. 1;





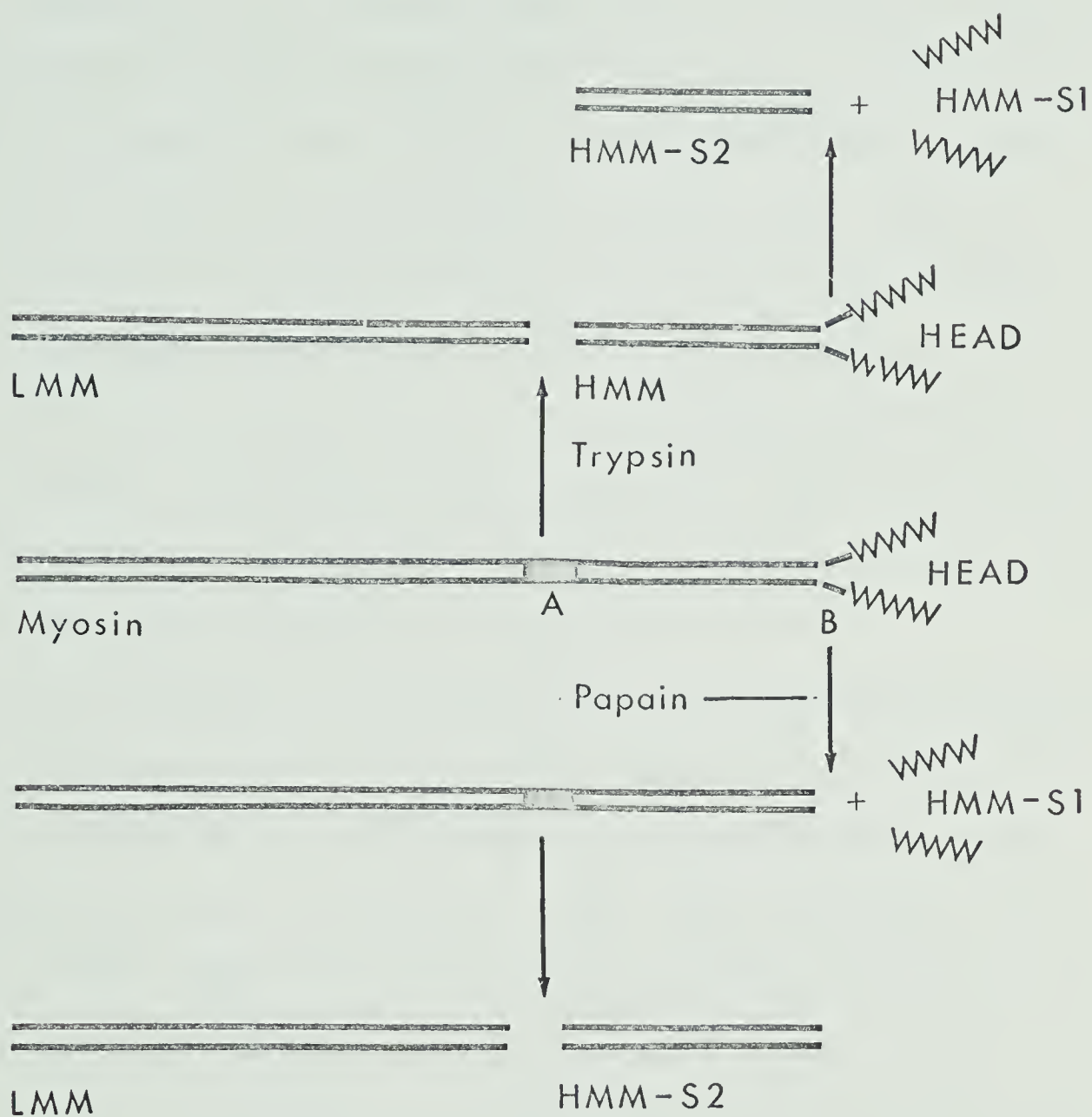
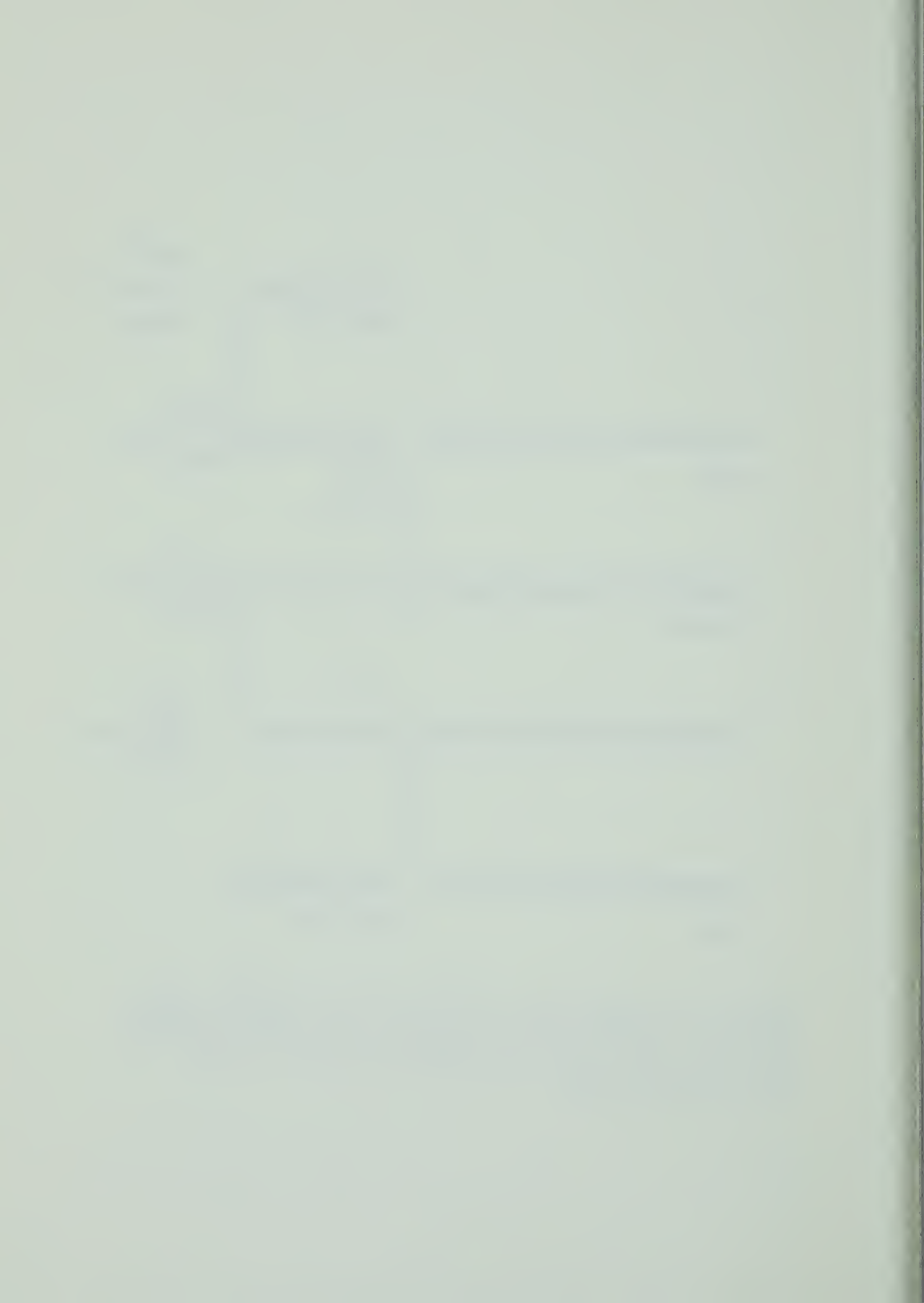


Figure 1: The proteolytic digestion scheme for myosin. Heavy meromyosin and light meromyosin result from a limited tryptic digestion, followed by further digestion into HMM-S1 and HMM-S2. Light meromyosin, HMM-S1, and HMM-S2 result from a single digestion with papain.



Cohen, 1961; Lowey and Cohen, 1962; Huxley, 1963; Rice et al, 1966; Slayter and Lowey, 1967). Within the myosin molecule are regions which are highly susceptible to proteolytic digestion such that two main fragments, heavy meromyosin (HMM) from the head region (Slayter and Lowey, 1967) and light meromyosin (LMM) result from a limited tryptic digestion (Gergely, 1953; Mihalyi and Harrington, 1959). Further digestion of HMM with trypsin (Mueller and Perry 1961; 1962) or a single digestion with papain (Kominz et al, 1965) gives an ATPase active subfragment (HMM-S1) with a mass of 115,000 daltons. The HMM-S2 depicted in this diagram is believed to be a water soluble helical fragment from the tail portion of HMM (Lowey et al., 1969) which appears as a slow peak in the sedimentation analyses of crude papain digests (Kominz et al., 1965; Lowey et al., 1969).

On the basis of the recoverable mass of the ATPase active material after digestion, it has been repeatedly suggested that there are two or three HMM-S1 units in each HMM unit or in one molecule of myosin as depicted in Fig. 1 (Young et al., 1965; Nauss and Gergely, 1967; Nihei and Kay, 1968). Electron microscopy has also been used to demonstrate two-headed myosin structures (Rice et al., 1966; Slayter and Lowey, 1967). In addition, it has been shown that both HMM and myosin contain two molecules of 3-methylhistidine to the one in HMM-S1 (Johnson et al., 1967). One mole of myosin binds two moles of ADP, or ATP, or pyrophosphate (Kiely and Martonosi, 1968; Schiselfeld and Bárány, 1968; Lowey and Luck, 1969; Nauss et al., 1969; Eisenberg and Moos, 1970), in contrast to a single binding site in subfragment (Young, 1967a; Nauss et al., 1969; Eisenberg and Moos, 1970). This



information supports the earlier idea that the two or three major subunits of myosin terminate in globular subfragments (Young et al., 1964).

Thus, by proteolytic digestion, the two centers for ATPase and actin-combination can be separated from the parent molecule. The HMM-S1 is soluble in water, while the light meromyosin swells into a hydrated gel, retaining the solubility characteristics of myosin (Mihalyi and Szent-Györgyi, 1953).

When myosin is bound with actin monomer or F-actin, the ATPase activity changes its response to the effect of  $Mg^{2+}$  (Banga, 1943; Banga and Szent-Györgyi, 1943). As it was first observed by Szent-Györgyi (1941, 1942), actomyosin gel at low ionic strength ( $<0.1$ ) and in the presence of  $Mg^{2+}$  (0.1mM) undergoes an intensive syneresis by the addition of ATP, forming a densely packed precipitate (superprecipitation). Systematic studies on the relationship between these processes were initiated by Weber (1958) who pointed out the possibility of superprecipitation being a simple model of muscular contraction.

Regarding the effect of  $Mg^{2+}$  on the actin-myosin interaction, two different explanations have been proposed: (1) the formation of  $MgATP^{2-}$  complex promotes the ATPase and superprecipitation (Watanabe and Yasui, 1965); and (2) a binding reaction of  $Mg^{2+}$  to the protein is responsible for the activation (Geske et al., 1957; Nihei et al., 1966, Nihei, 1967). In the latter proposal it seemed unlikely that the superprecipitation occurred as a mere consequence of the effect of  $Mg^{2+}$  on the actomyosin ATPase, since the concentrations of  $Mg^{2+}$  required to observe the maximum rate of superpre-





cipitation are well above that needed for the maximum activation of ATPase (Nihei, 1967). In either of the above cases, the proposal has not been tested by direct measurements of binding reactions.

Previously, it was thought that under certain ionic conditions,  $\text{Ca}^{2+}$  is a necessary factor for the interaction of actin and myosin in the presence of ATP and  $\text{Mg}^{2+}$  (Weber, 1959). The requirement for  $\text{Ca}^{2+}$ , however, was explained to be due to tropomyosin and troponin which jointly act as an inhibitory factor of the actin-myosin interaction. The inhibitory effect of these proteins is cancelled by adding  $\text{Ca}^{2+}$  to the system (Ebashi, 1963; Ebashi and Ebashi, 1964; Ebashi and Kodama, 1966). The same workers have established, however, that with carefully prepared actomyosin, these effects are abolished (see also Perry et al., 1966). Using such preparations, it remains to be tested whether  $\text{Ca}^{2+}$  can replace  $\text{Mg}^{2+}$  in the reaction of ATP and actomyosin leading to superprecipitation.

The myosin HMM-S1, like the whole molecule, combines with the actin monomer or F-actin and results in increased  $\text{Mg}^{2+}$ -ATPase such as to suggest that this property was unadulterated by digestion procedures (Eisenberg et al., 1968). It is generally accepted from ultracentrifugal (Young, 1967b) and ATPase measurements in the presence of  $\text{Mg}^{2+}$  (Young, 1967a; Rizzino et al., 1968), that the actin monomers of F-actin HMM-S1 combine in a mole to mole ratio.

Contrary to the idea of two actin-combining sites in myosin or heavy meromyosin, Young (1967b) and Rizzino et al., (1970) also found a mole per mole association of the actin monomer to heavy meromyosin. The electron microscopy of Huxley (1963) had forwarded this proposal at an earlier date. However, Szentkiralyi and





Oplatka (1969), studying the increase in the  $Mg^{2+}$ -ATPase of HMM when it combines with F-actin, reported that two actin monomers combine with HMM, the second binding being weaker and occurring only at high concentrations of F-actin. Tawada and Oosawa (1969) prepared copolymers and homopolymers of carboxymethylated and natural actin and used them to form modified actomyosin. Their experiments showed that two actin monomers per mole of myosin are required to give the full ATPase of actomyosin. There exists, therefore, a need to clarify the binding reaction between actin and myosin.

The hydrated gel of actomyosin at low KCl concentrations consists of myosin and actomyosin in the form of filaments (Huxley, 1963). These filaments are of the order of microns in length so that it is not unreasonable to view the hydrated actomyosin as a network of actin and myosin filaments (Eisenberg and Moos, 1967) with water in the interfilamentous space. During superprecipitation, the actomyosin gel apparently expels trapped water (Szent-Györgyi, 1951). Recently, a light scattering study revealed changes which were interpreted as being due to a density change (Nihei and Yamamoto, 1969). It was not certain, however, whether just the shrinking of actomyosin was involved or whether there was a conformational alteration in either actin or myosin. It has not been examined whether any physical change can be detected in a mixture of actin and subfragment under conditions favourable for superprecipitation. This actin-subfragment system, which is equivalent to actomyosin except that it lacks the light meromyosin portion, might be useful for investigating the functional significance of the deleted part of myosin.



## II. METHODS AND MATERIALS

### 1. The Preparation of Myosin and Actin

#### A. The Preparation of Myosin

Myosin was prepared from the back and leg muscles of albino rabbits by the procedure of Tonomura et al., (1966). After the excision of fat deposits and connective tissue, the muscle was homogenized in 3 volumes of a 0.3M KCl solution containing 0.2mM ATP and 0.02M histidine (pH 6.8), in the stainless steel vessel of the Sorvall Omni-mixer (Sorvall Inc., Norwalk, Conn., U.S.A.). The homogenate, after standing for 10 minutes, was centrifuged for 20 minutes at 6,000 rev./min. in the GSA rotor of a Sorvall RC2-B centrifuge. The supernatant was filtered through 4 layers of clean gauze into a chilled graduate cylinder. The volume of filtrate was estimated and this was added to 9 volumes of double distilled water at 4°C in a large conical container with a clamped opening at the bottom. The precipitated crude myosin sedimented in approximately 45 minutes. The sedimentation was sometimes prolonged and seemed to depend upon fat content and the proportion of leg muscle used. The precipitate was collected from the bottom and centrifuged at 6,000 rev./min. for 20 minutes. The packed precipitate was resuspended in a buffer solution (0.5M KCl, 0.02M histidine, pH 6.8). Then, it was diluted with double distilled water to make the suspension in 0.28M KCl. This was stirred for at least 30 minutes to ensure breaking up of lumps and dissolving the myosin. Insoluble material was removed by centrifugation at 60,000 x g for 30 minutes in the A-211 rotor of an International B-60 preparative



ultracentrifuge (International, Needham Heights, Mass., U.S.A.). The supernatant was passed through 4 layers of gauze and myosin was precipitated at 0.05M KCl. The precipitate was again collected by centrifuging at 6,000 rev./min. for 20 minutes. The dissolution-precipitation cycle was repeated except that the second dissolution was at 0.25M KCl. The myosin prepared showed no turbidity change under the conditions for superprecipitation and no  $Mg^{2+}$  activation of the ATPase activity, both indicating the absence of actomyosin contamination. The final myosin precipitate was resuspended in a small volume of 0.5M KCl, 0.02M histidine (pH 6.8), and dialysed against a large volume of the same. Before use this myosin solution was centrifuged at 100,000 x g for 2-3 hours to separate denatured material and fat. The stock myosin solution usually contained 30-50 mg/ml. Table I shows the  $Ca^{2+}$ -ATPase activity of several preparations (0.05M KCl, 0.05M tris-Cl, pH 7.4, 10mM  $CaCl_2$ , 1.0mM ATP, 0.12 mg/ml, 25°C). The average activity of 0.56  $\mu$ mole Pi/min.mg agrees with that of other workers (Lowey et al., 1969). The observed deviation is likely due to a variation of denatured material in addition to the error of measurement. The state of the myosin sulfhydryl groups did not appear to be severely affected because the p-mercuribenzoate effect was consistent among the preparations tested. The enzymatic activity of myosin was stable for at least two weeks (prep 2 and 3).

Some preparations were stored in 50% glycerol for 1-3 months before use. Pre-cooled glycerol was added slowly with stirring to a measured volume of myosin solution, the presence of air bubbles being avoided. The container was subsequently sealed and stored at -25°C.



Table 1  
Ca<sup>2+</sup>-ATPase Activity of Several Preparations  
of Myosin

Date of Preparation	Ca <sup>2+</sup> -ATPase (μmoles Pi /min. mg)	
	Untreated	pMB-modified*
1. (Oct /66)	0.43	1.05 (2.1)
2. (Nov 17/66)	0.42	1.18 (2.5)
(Nov 22/66)	0.44	---
(Nov 28/66)	0.39	---
3. (Nov 30/66)	0.45	1.25 (2.5)
(Dec 12/66)	0.48	---
4. (Dec /66)	0.52	1.12 (2.5)
5. (Apr /67)	0.62	---
6. (May /67)	0.61	---
7. (Aug /67)	0.46	---
8. (Sept /67)	0.51	---
9. (Jan /68)	0.43	---
10. (Mar /68)	0.56	---
11. (Oct /68)	0.54	---
12. (Nov /68)	0.65	---
13. (Dec /68)	0.86	---
14. (Jan /69)	0.97	---
15. (June /69)	0.61 (glycerol stored)	---
16. (Aug /69)	0.44	---
17. (Dec /69)	0.41	---
	Mean = 0.56 ± 0.16	

ATPase: 0.05M KCl, 0.05M tris-Cl (pH 7.4), 10mM CaCl<sub>2</sub>,  
1.0mM ATP, 0.12 mg myosin/ml, 25°C.

\*In brackets is shown the added pMB in moles/10<sup>5</sup> g  
required to give the maximum ATPase indicated.







For the removal of glycerol, the myosin-glycerol mixture was equilibrated to 0°C, and dialysed against 0.5M KCl, 0.02M histidine (pH 7.4). Care was taken to immerse only the lower 50% of the dialysis bag in the buffer to create an outward hydrostatic pressure opposing the inward osmotic pressure which may otherwise burst the bag. It was confirmed that glycerol storage did not adversely affect the ATPase activity (prep. 15).

#### B. Chromatographic purification of myosin

Certain myosin preparations were further treated by passing them through a column of cellulose phosphate and DEAE-cellulose (Diethyl Amino Ethyl Cellulose) (Sigma Chemical Co., St. Louis, Mo., U.S.A.) in order to remove impurities of AMP deaminase and nucleic acid.

The procedure presented here is essentially that of Harris and Suelter (1967), and was simplified to obtain an improved yield. The cellulose phosphate (0.61meq/gm, medium mesh) and DEAE-cellulose (0.80meq/gm, medium mesh) were both washed successively with 0.5M NaOH, 0.5M HCl, 0.5M NaOH, and finally with 5mM EDTA solution. The cellulose was washed with distilled water after each of these washes until an indicator reaction showed a pH near neutral. These ion exchangers were subsequently washed with the eluting buffer (0.2M KCl, 0.02M tris-Cl, pH 7.4) and the suspensions were degassed under reduced pressure for 24 hours before being poured into the column. The column was made by layering cellulose phosphate (50 cm) on top of DEAE-cellulose (30 cm) in a 2.5 x 100 cm column (Pharmacia, Uppsala, Sweden). The two exchangers were thus arranged in tandem with minimal mixing at the interface. The column was fitted with the flow



adapters and then equilibrated with the buffer. Approximately 1 g of myosin at a concentration of 10 mg/ml was pumped through Tygon tubing of small diameter (1mm) onto the top of the column with an LKB 4912A peristaltic pump (LKB-produkter AB, Stockholm, Sweden). The column was then eluted with the buffer at a flow rate of 32 ml/hr. The eluant was monitored with a LKB Uvicord (type 8301A) at 254 m $\mu$ . Fractions were automatically collected at a rate of 3 fractions/hr. in an LKB Ultrorac fraction collector (type 7000). Each fraction was then analysed for protein concentration by the biuret reaction; the nucleic acid contamination using the ratio of optical densities measured at 280 and 260 m $\mu$  (Layne, E., in Methods of Enzymology, Vol. 3, 1957); the Ca<sup>2+</sup>-ATPase activity in a reaction mixture containing 0.05M KCl, 0.05M tris-Cl (pH 7.4), 10mM CaCl<sub>2</sub>, 1mM ATP, 25°C; and the AMP deaminase activity as described below. Previous studies of DEAE-cellulose chromatography have indicated an elution pattern similar to that shown in Fig. 2 (Perry, 1960; Asai, 1963; and Richards et al., 1967). The leading protein peak showed a higher Ca<sup>2+</sup>-ATPase activity than did the trailing proteins. Approximately 50% of myosin applied on the column was recovered free of AMP-deaminase activity (hatched area, Fig. 2). The column treatment gave protein fractions completely free from nucleic acid contamination.

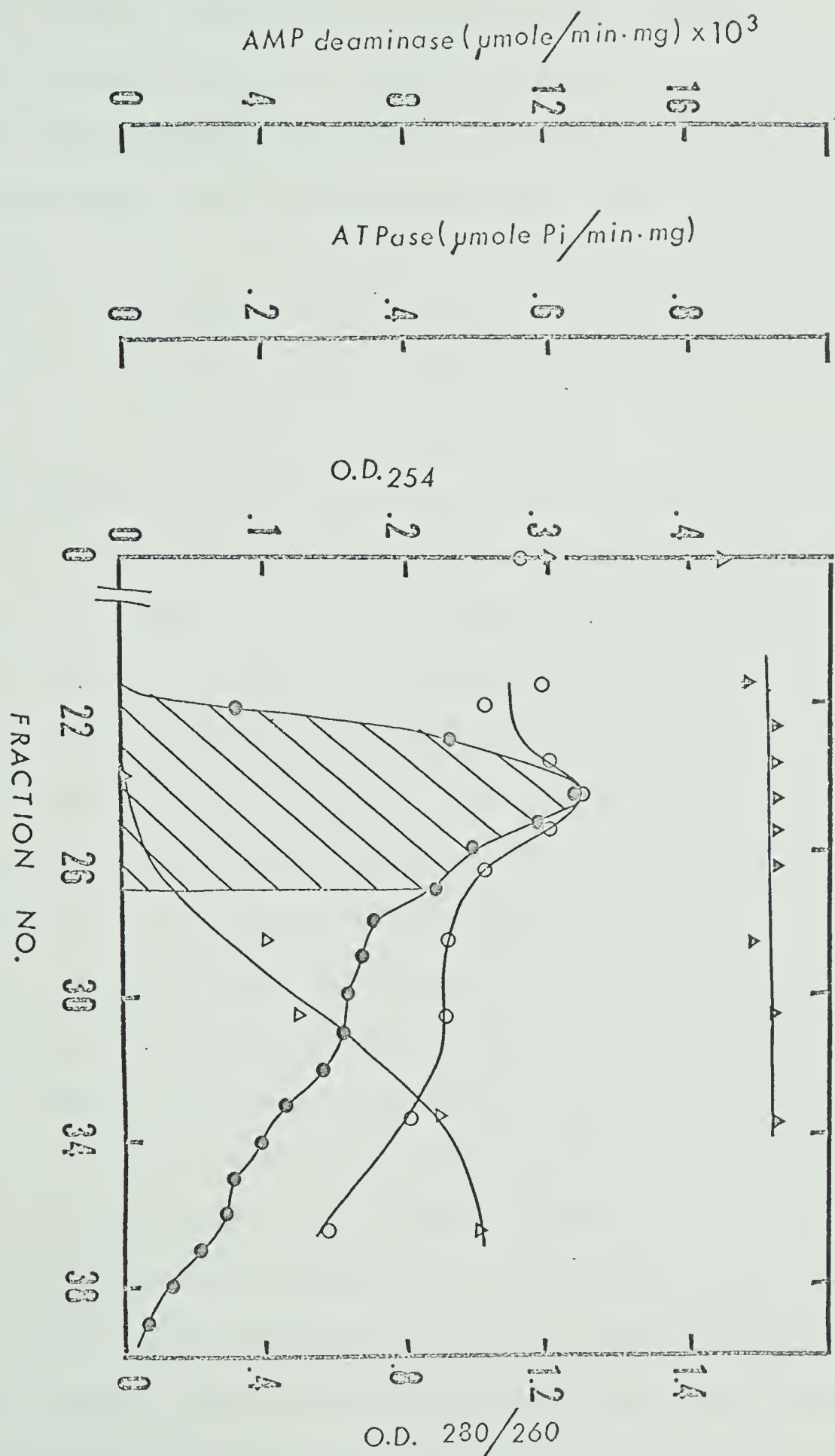
#### C. The preparation of an enzymatically active subfragment of myosin

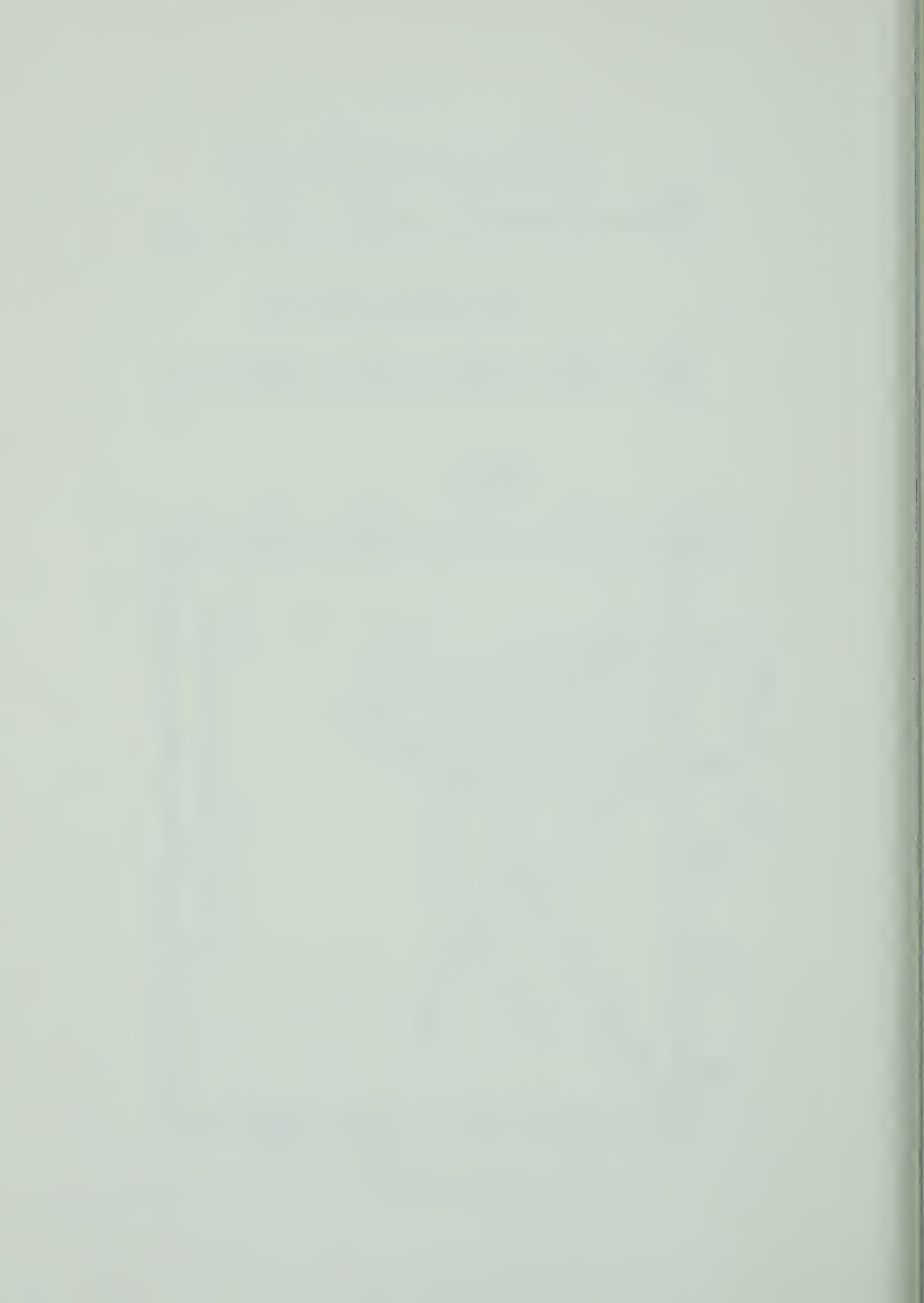
Myosin was digested with an insoluble papain-cellulose complex (Kominz et al., 1965) and the ATPase active fragment was subsequently isolated and purified by gel-exclusion chromatography or by forming its complex with F-actin (Mueller and Perry, 1962). Papain (100 mg



Figure 2: The cellulose phosphate and DEAE-cellulose chromatography of myosin. The myosin was eluted with 0.2M KCl, 0.02M tris-Cl (pH 7.4), 4°C. (●), absorbance at 254 mμ; (○), Ca<sup>2+</sup>-ATPase at 0.05M KCl, 0.05M tris-Cl (pH 7.4), 10mM CaCl<sub>2</sub>, 1.0mM ATP, 0.12 mg myosin/ml, 25°C; (Δ), AMP deaminase at 0.1M KCl, 0.01M histidine (pH 6.5), 0.05mM AMP, 30°C; (▲), 280/260 absorbance ratio. On the ordinate is indicated the values for unchromatographed myosin.









of 2 x crystallized papain in sodium acetate, Sigma Chemical Co.) was suspended in 50 ml of 5mM EDTA containing 2mM cysteine (pH 7.4), and was then dissolved by adjusting the pH to 5-5.5 with 0.1N HCl. The dissolved papain was coupled to diazonium salt of p-aminobenzyl-cellulose (Bio-Rad Laboratories, Richmond, Calif., U.S.A.) by mixing it with 0.2 g of the cellulose in 50 ml of 0.05M  $K_2HPO_4$  buffer (pH 7.0). The diazotized cellulose was prepared according to the procedure of Campbell et al., (1951). After being stirred slowly overnight at 4°C, the papain-cellulose complex was washed 3 times by centrifugation in the cold with 2mM EDTA, 5mM cysteine (pH 7.4), to rid it of uncombined papain and fine cellulose particles. The cellulose complex was finally suspended in an equal volume of the same solution. The activity of papain-cellulose was standardized by determining the rate of protein release from myosin (5-10 mg/ml) in a reaction mixture containing 0.5M KCl, 1mM dithiothreitol (D.D.T.), and 1mM EDTA (pH 7.0), at 25°C, using a pH-stat method (Nihei and Kay, 1968). In this procedure the release of hydrogen ions during proteolytic hydrolysis was followed by adding 16mM NaOH to maintain the pH value of the solution using a Radiometer titrator, type TTTIc, an autoburette, type ABU/a, and a titrigraph, type SBR 2C. The air phase in the reaction vessel was purged with ultra-pure Nitrogen (Linde, Union Carbide of Canada) to avoid CO<sub>2</sub> contamination. The quantity of hydrogen was corrected for the protonation of  $\alpha$ -amino groups (Mihalyi and Harrington, 1959). For the preparative purpose, a large quantity, ~500 mg, of myosin was then digested under the same condition except that 0.02M histidine was present as a



buffer (pH 7.0). The reaction was terminated at intervals by centrifugation in a clinical centrifuge and by ultrafiltration through a Millipore filter of pore size  $1.2\ \mu$ . The digested myosin was dialysed against  $0.01\text{M K}_2\text{HPO}_4$  buffer (pH 7.4), to a final concentration of  $0.05\text{M KCl}$ , and the insoluble fraction was removed by centrifugation at  $100,000 \times g$  for one hour. The supernatant was concentrated against a bed of Ficoll (Pharmacia) or by filtering through an Amicon Centriflo cone (Amicon Corp., Lexington, Mass., U.S.A.) at  $3,000\ \text{rev./min.}$  for 30 minutes in the Sorvall SS-34 rotor (Eisenberg et al., 1968). This concentrate (approx. 3 ml) was applied by means of a LKB peristaltic pump to a  $2.5 \times 30\ \text{cm}$  column of G-200 superfine Sephadex (Pharmacia) which had been pre-equilibrated with  $0.05\text{M KCl}$ ,  $0.01\text{M K}_2\text{HPO}_4$  (pH 7.4) and  $1\text{mM}$  dithiothreitol (D.T.T.). The column was eluted in a descending fashion with the same buffer solution at a flow rate of  $3\ \text{ml/hr.}$  The eluant was monitored using an optical density at  $254\ \text{m}\mu$  and was collected in fractions by the LKB apparatus. The fractions containing the active subfragment were pooled and mixed with a quantity of F-actin. The actin-fragment complex was subsequently collected at  $100,000 \times g$  for 2-3 hours. The pellet was resuspended with the aid of a Teflon pestle in  $5\text{mM NaPP}$ ,  $1\text{mM MgCl}_2$ ,  $0.05\text{M KCl}$ ,  $0.01\text{M K}_2\text{HPO}_4$  (pH 7.4), and centrifuged at  $100,000 \times g$  for 2-3 hours. The supernatant containing the subfragment was concentrated against Ficoll and was then dialysed against a large volume of  $0.05\text{M KCl}$ ,  $0.01\text{M K}_2\text{HPO}_4$  (pH 7.4),  $1\text{mM}$  Dithiothreitol (D.T.T.). This stock solution was centrifuged at  $100,000 \times g$  for 1 hour to remove a precipitate. All operations were carried out in the cold.



The fragment was used within one week or stored by freezing at  $-25^{\circ}\text{C}$ . The enzymatic activity was periodically checked at 0.05M KCl, 0.05M tris-Cl (pH 7.4), 10mM  $\text{CaCl}_2$ , 1.0mM ATP,  $25^{\circ}\text{C}$ .

D. The preparation of natural actomyosin

Like myosin, actomyosin was extracted from the back and leg muscles of albino rabbits according to Szent-Györgyi (1951). This muscle was put through a meat grinder after removal of fat deposits and connective tissue and then mixed with 3 volumes of Weber-Edsall solution (0.6M KCl, 0.01M  $\text{Na}_2\text{CO}_3$ , 0.01M  $\text{NaHCO}_3$ ). After a 24 hour extraction in the cold the highly viscous mixture was diluted with an equal volume of Weber-Edsall solution and centrifuged at 6,000 rev./min. for 20 minutes in a Sorvall RC2-B centrifuge. The supernatant containing crude actomyosin was filtered through 4 layers of gauze into a graduate cylinder. By adding enough cold double distilled water to the filtrate to give a final KCl concentration of 0.25M, actomyosin was precipitated. The precipitate was collected by centrifugation at 6,000 rev./min. for 20 minutes and then redissolved in 0.6M KCl. This solution was centrifuged at 30,000 x g for 30 minutes to remove insoluble debris. The supernatant was filtered again, precipitated, and redissolved in 0.6M KCl. After the final centrifugation, the middle layer of the centrifuged solution was collected avoiding fat contamination. Actomyosin gel was washed with 0.005M tris-Cl (pH 7.4), in order to make the  $\text{Mg}^{2+}$ -ATPase activity EGTA-insensitive (Perry et al., 1966). After the second wash the actomyosin suspension was left standing in this tris buffer for 72 hours, after which it was centrifuged at 6,000 rev./min.



for 20 minutes, redissolved in 0.6M KCl, 0.02M histidine (pH 7.4), and then dialysed against a large volume of the same in the cold. In Table 2 the washed actomyosin was compared to a normal preparation for EGTA sensitivity, and it is shown that washing makes actomyosin EGTA insensitive. Apparently the troponin-tropomyosin system has been removed by this treatment (Perry et al., 1966; Ebashi and Endo, 1968).

#### E. Modification of the sulfhydryl groups of myosin

##### 1) pMB-modified myosin

Myosin at a concentration of 1.33 mg/ml was incubated for 5 minutes at zero degrees in the presence of an appropriate amount of pMB. Dependence of the enzymatic activity on the pMB concentration showed an activation and inhibition as observed by Kielly and Bradley (1956). Maximum activation occurred in the presence of 2.5 moles of pMB/ $10^5$  g as was also shown by Mattocks et al. (1967). The activity was analysed at 0.05M KCl, 0.05M tris-Cl (pH 7.4), 10mM  $\text{CaCl}_2$ , 1.0mM ATP, 0.12 mg myosin/ml, 25°C.

##### 2) NEM-modified myosin

Myosin at a concentration of 10 mg/ml was treated with 2.0 moles of N-ethylmaleimide (NEM)/ $10^5$  g of myosin for 30 minutes (Perry and Cotterill, 1965). The reaction was stopped by the addition of a 50 fold excess of  $\beta$ -mercaptoethanol (Sekine and Kielley, 1964). The increase in the activity was analysed at 0.4M KCl, 0.05M tris-Cl (pH 7.4), 10mM  $\text{CaCl}_2$ , 1.0mM ATP, 0.12 mg myosin/ml, 25°C.

#### F. Extraction and purification of actin

The acetone powder of muscle (Straub, Szent-Györgyi, 1951) was





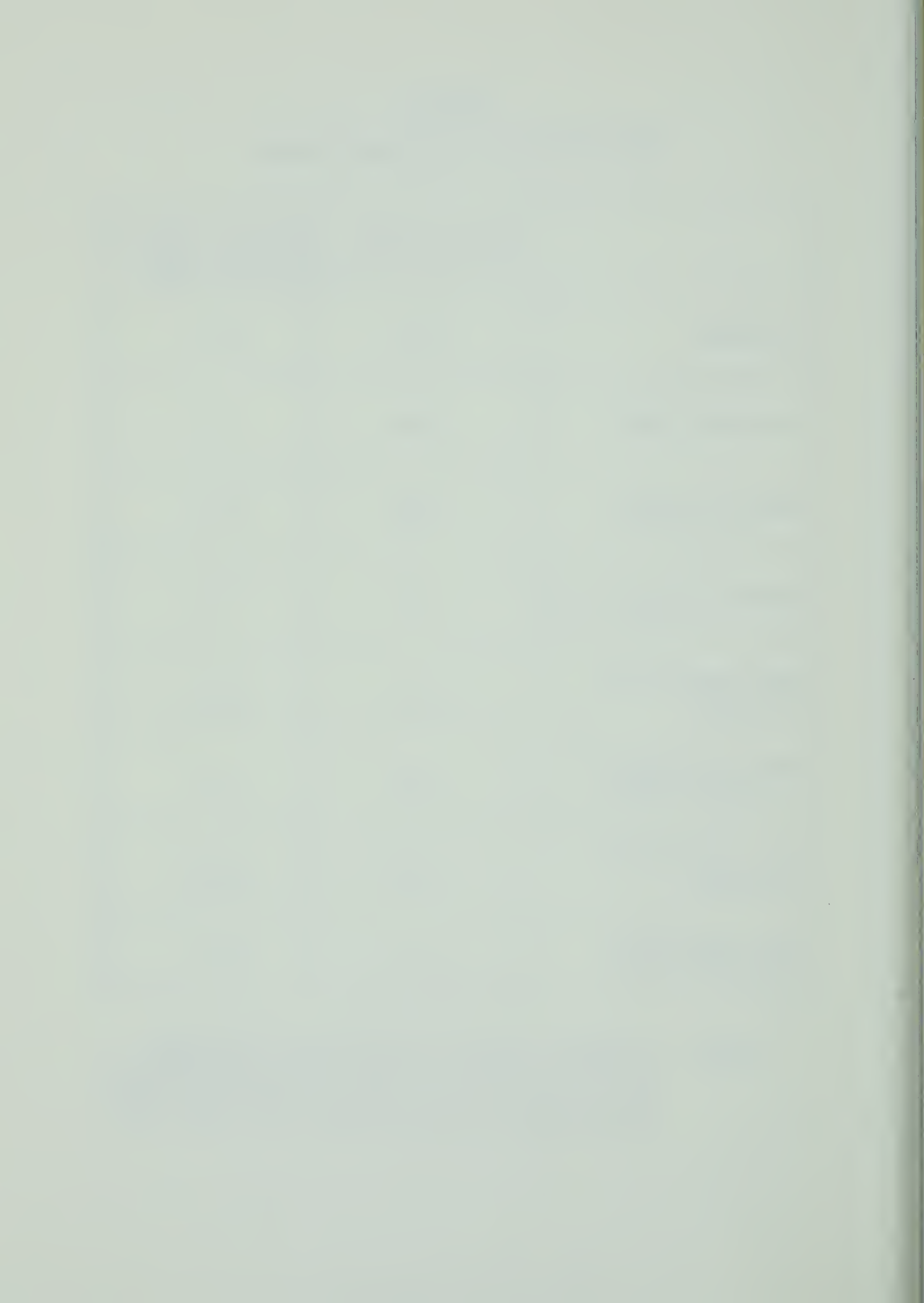


Table 2

EGTA Sensitivity of Actomyosin Samples

	ATPase activity ( $\mu$ moles Pi/min. mg)	Percent of maximum activity in the absence of EGTA
Actomyosin	0.147	100
Actomyosin + EGTA	0.087	59
Washed actomyosin	0.188	100
Washed actomyosin + EGTA	0.174	92
Crude reconstituted actomyosin	0.557	100
Crude reconstituted actomyosin + EGTA	0.378	68
Pure reconstituted actomyosin	0.218	100
Pure reconstituted actomyosin + EGTA	0.232	106

ATPase: 0.006M KCl, 0.05M tris-Cl (pH 7.4), 2.0mM MgCl<sub>2</sub>,  
1.0mM ATP, 1mM EDTA, 1mM EGTA, 0.06 mg actomyosin/ml,  
0.06 mg washed actomyosin/ml, 0.20 mg reconstituted  
actomyosin/ml (3:1 actin: myosin molar ratio), 25°C.



usually prepared from ground (not homogenized) back and leg muscle after the myosin extraction. The muscle was first suspended with constant stirring in 5 volumes of a 0.4%  $\text{NaHCO}_3$  solution at room temperature for 30 minutes. The fluid was expressed through 4 layers of gauze and the residue suspended in one volume of a solution of chilled 0.01M  $\text{NaHCO}_3$  and 0.01M  $\text{Na}_2\text{CO}_3$  for 10 minutes. Then the suspension was diluted with 10 volumes of distilled water at room temperature, and immediately strained through gauze. Three volumes of cold acetone were mixed with the residue at a temperature below  $25^\circ\text{C}$ . After stirring for 10 minutes, the acetone was squeezed out and this procedure repeated. The final residue was left to dry at room temperature and the dry fiber was stored at  $-25^\circ\text{C}$  in a sealed container.

The extraction of actin from the fiber was done with 20 volumes of 0.2mM ATP, 3mM cysteine (pH 8.0), hereafter called the "depolymerizing solution" for 20 minutes at  $0^\circ\text{C}$  in order to minimize the tropomyosin contamination (Drabikowski and Gergely, 1962). The first phase of actin purification used the polymerization-depolymerization cycle of Mommaerts (1951). The extracted actin was polymerized with 0.1M KCl, 1mM  $\text{MgCl}_2$ , overnight at  $4^\circ\text{C}$  or at room temperature for 1 hour, and then centrifuged at  $120,000 \times g$  for 1 hour in an International A-211 rotor. The sides of the centrifuge tubes and the pellets were rinsed with cold water to get rid of fat and excess salt. Then the pellet was suspended in a small volume of the depolymerizing solution (100 ml if 50 g of fiber was originally extracted), and homogenized in a Teflon-glass homogenizer. This



suspension was dialysed overnight against 20 times its volume of depolymerizing solution, followed by centrifugation at 120,000 x g. The resulting G-actin was aspirated from the central portion of the solution to avoid fat. This depolymerization-polymerization cycle was repeated.

The second phase of actin purification involved a partial polymerization step to further eliminate tropomyosin contamination (Martonosi, 1962; Laki et al., 1962). The F-actin was depolymerized as before, except that it was suspended in a smaller volume of depolymerizing solution (30 ml for 50 g of originally extracted fiber) so as to maintain the actin concentration above the critical value (Oosawa et al., 1959) for polymerization (2.5 mg/ml seemed to be ideal). This G-actin was polymerized overnight by dialysis against 0.7mM  $\text{MgCl}_2$ , 0.4mM ATP, 3mM cysteine (pH 8.0). The partially polymerized actin was centrifuged at 70,000 x g for 3 hours. Under these conditions, the tropomyosin contaminated actin was difficult to polymerize, and remained in the supernatant. The pure F-actin sedimented, forming a soft pellet. This polymerization cycle was also repeated. Finally the actin was polymerized with 0.1M KCl, 1mM  $\text{MgCl}_2$  and dialysed against 0.1M KCl to remove free  $\text{Mg}^{2+}$ . The low viscosity of actin (Drabikowski and Gergely, 1962) and the lack of a hypersharp peak in the sedimentation of EDTA-denatured actin (Laki et al., 1962), indicated that actin prepared in this way is tropomyosin-free. In Table 2, the lack of an EGTA-sensitivity of reconstituted actomyosin gives an index of purity.



## 2. Estimation of Protein Concentration

The concentrations of actin, myosin and papain subfragment were routinely determined by the biuret method as standardized with human albumin (Dade Reagents Inc., Miami, Fla., U.S.A.), and calibrated against a micro-Dumas nitrogen determination (Coleman nitrogen analyzer, Model 29), assuming 16.5% nitrogen in myosin (Kominz et al., 1954) and actin (Rees and Young, 1967). Actin and papain subfragment were also determined at 280 m $\mu$  using an extinction coefficient of 1149 cm<sup>2</sup>/g (Estes and Moos, 1969) and 770 cm<sup>2</sup>/g (Young et al., 1965), respectively. The results obtained by the biuret and optical methods agree with each other within the experimental error.

## 3. Enzymatic Assays

### A. Nucleoside triphosphatase

#### 1) Molybdovanadate method for inorganic phosphate (Lecocq and Inesi, 1966)

The reaction was initiated by adding nucleoside triphosphate (NTP) to a constantly stirred mixture containing myosin and other components. Aliquots (1 ml each) of the reaction mixture were taken at intervals and added to 2 ml of the molybdovanadate reagent. The mixture was centrifuged to remove precipitated proteins and the absorbance at 400 m $\mu$  was determined after 10 minutes. A suppression of the colour was encountered when a high protein concentration was used. This difficulty was circumvented by adding 0.05  $\mu$ mole of K<sub>2</sub>HPO<sub>4</sub>/ml of reaction mixture. This was found to have no effect on the enzymatic rate determination.





## 2) Raabe method for inorganic phosphate (Raabe, 1955)

A description is given in the EM-test (E. Merck, Darmstadt, Germany; in the U.S.A., Brink Instruments, Westbury, N.Y.). An aliquot (1 ml each) was added to the molybdate reagent and the resulting phosphomolybdate was reduced to colloidal molybdenum blue. After fifteen minutes a sulfite-carbonate reagent was added to solubilize the protein, and after a further fifteen minutes the blue color was read at 750 mμ.

## 3) Assay of hydrogen ion release by pH Stat

A solution of 16mM NaOH was standardized with 0.1N HCl (Radiometer). The mixture of components was adjusted to the required pH with the standard NaOH (the titrating system was the same as described for the papain hydrolysis of myosin). The reaction chamber was maintained in a nitrogen (ultrapure, Linde) atmosphere. Myosin was added to initiate the reaction.

## B. Adenosine monophosphate deaminase

Myosin was added to a constantly stirred mixture containing 0.1M KCl, 0.01M histidine (pH 6.5), and 0.05mM adenosine monophosphate (AMP) in a thermostated cuvette of 2 cm light path. The specific activity of AMP deaminase (μmoles/min.mg of myosin) was determined in a Zeiss PMQ II spectrophotometer, using the molar coefficient difference between AMP and IMP at 265 mμ of  $8.86 \times 10^3$  l/cm. mole (Harris and Suelter, 1967).

## 4. The Binding Reactions of Calcium and Magnesium to Nucleoside Triphosphates, and Actin and Myosin



A. Preliminary treatment of the proteins and reagents with a chelating resin.

F-actin and myosin and all reagents were treated with chelex-100 (California Biochemical Corp., Los Angeles, Calif., U.S.A.) to decrease contaminating cations (Offer, 1964). Myosin was dialysed against a large volume of a solution in which chelex-100 was suspended (usually 100 ml of settled chelex in 1 litre of solution), or by passing myosin through a 2.5 x 15 cm column of chelex (Offer, 1964) which had been pre-equilibrated with 0.5M KCl, 0.02M histidine (pH 7.4). F-actin was dialysed against the chelex suspension or against an EDTA solution, the EDTA being subsequently removed by repeated dialysis. KCl, tris-Cl,  $\text{NH}_4\text{OH}$ , and ATP were purified by similarly passing them through a column of chelex which was in the  $\text{K}^+$ ,  $\text{H}^+$ ,  $\text{NH}_4^+$ , and  $\text{Na}^+$  form, respectively. The ability of the resin to remove trace quantities of divalent cations was tested using radioactive  $\text{Ca}^{2+}$  (Table 3). The  $\text{Ca}^{2+}$  contamination of myosin and of chelex-100 was determined by the fluorometric technique of Kepner and Hercules (1963) using calcein. For this experiment,  $\text{Ca}^{2+}$  was extracted from myosin or chelex (2 ml) with 7% (w/v) trichloroacetic acid (TCA) for 24 hr. at room temperature (Mori, 1959). The TCA extract, separated from the proteins by centrifugation, was dried in a sandbath at  $75^\circ\text{C}$  and placed overnight in a desiccator containing a bed of NaOH pellets to remove TCA. The calcein solution (Phillips, 1964) was added directly to the residue and the fluorescence was determined in a Turner Model 110 fluorometer. The results indicated an incomplete removal of  $\text{Ca}^{2+}$  from myosin (Table 4).



Table 3

The Calcium Purification of Reagents by Chelex-100

Reagent	$^{45}\text{Ca}^{2+}$ cpm		Background	% Purification
	Untreated	Treated		
20mM ATP (~30 ml)	158.7	11.6	9.9	≈100
1M KCl (~300 ml)	205.4	38.5	39.8	≈100
0.5M Tris (~200 ml)	71.8	9.4	9.7	≈100

Radioactive  $^{45}\text{Ca}^{2+}$  was added to each reagent (0.01-0.02  $\mu\text{C}$   $^{45}\text{CaCl}_2$ /10 ml) which was subsequently treated with chelex-100 in a 2.5 x 15 cm column. The radioactivity of 1 ml aliquots was determined in a Tracerlab ultrathin window gas flow counter with 4% efficiency.



Table 4

Calcium Content in Myosin Before and After

Treatment with Chelex-100

Preparation	Chelex Treatment		Ca <sup>2+</sup> in myosin (moles of Ca <sup>2+</sup> ) (mole of myosin)	
	Ca <sup>2+</sup> lost from myosin (μmoles)	Ca <sup>2+</sup> gained by chelex 100 (μmoles)	Untreated	Treated
1. (160 mg.)	0.33	0.30	1.2	0.24
2. (250 mg.)	0.35	0.25	0.90	0.11

Myosin in 0.5M KCl, 0.02M histidine (pH 7.4) was dialyzed against 30 ml of chelex in this buffer. The effect of chelex treatment is indicated by the loss of Ca<sup>2+</sup> from myosin and the gain by the chelex.





In a separate experiment 1.4  $\mu$ moles of  $\text{Ca}^{2+}$  containing 0.02  $\mu$ c  $^{45}\text{Ca}^{2+}$  was added to 10 ml of myosin (2.6 mg/ml) at  $0^{\circ}\text{C}$ , which was then treated with a large volume of chelex. The radioactivity remained in myosin and revealed that 0.1 mole  $\text{Ca}^{2+}$  per mole myosin was exchangeable. It was assumed therefore, that the residual  $\text{Ca}^{2+}$  bound to myosin should be treated as a part of the bound  $\text{Ca}^{2+}$  which dissociates as the total  $\text{Ca}^{2+}$  concentration decreases.

The TCA extract of chelex-treated myosin and EDTA treated F-actin was also tested with calmagite (this procedure will be described in detail in the section on the  $\text{Mg}^{2+}$  binding to actin and myosin using calmagite). An aliquot (20 ml each) of 0.1mM calmagite in  $\text{NH}_4\text{OH-NH}_4\text{Cl}$  buffer ( $\mu=0.4$ , pH 10) was added to the dried TCA extract and the absorbance difference between 0.1mM calmagite and each sample,  $-\Delta \text{O.D.}$ , was measured at 640 m $\mu$ . From Table 5 it is seen that there is no detectable contamination of magnesium or other cations reacting with calmagite within the limits of experimental error. From the estimations of  $\text{Ca}^{2+}$  contamination in myosin and  $\text{Mg}^{2+}$  contamination in myosin and actin, the uncertainty of binding measurements was estimated as 0.1-0.2 mole per mole of myosin.

#### B. Calcium ( $^{45}\text{Ca}^{2+}$ ) binding to myosin using an ultrafiltration technique

The  $\text{Ca}^{2+}$  binding reaction was measured by ultrafiltration under the same conditions as for the enzymatic activity. The  $^{45}\text{Ca}^{2+}$  (535  $\mu$ c/82.4  $\mu$ g of  $\text{Ca}^{2+}$  in 1.4 ml; The radiochemical Centre, Amersham, Bucks., U.K.) was diluted before use. A 10 ml suspension of myosin in 0.05M KCl, 0.05M tris-Cl (pH 7.4), containing  $^{45}\text{Ca}^{2+}$  (0.01  $\mu$ c)

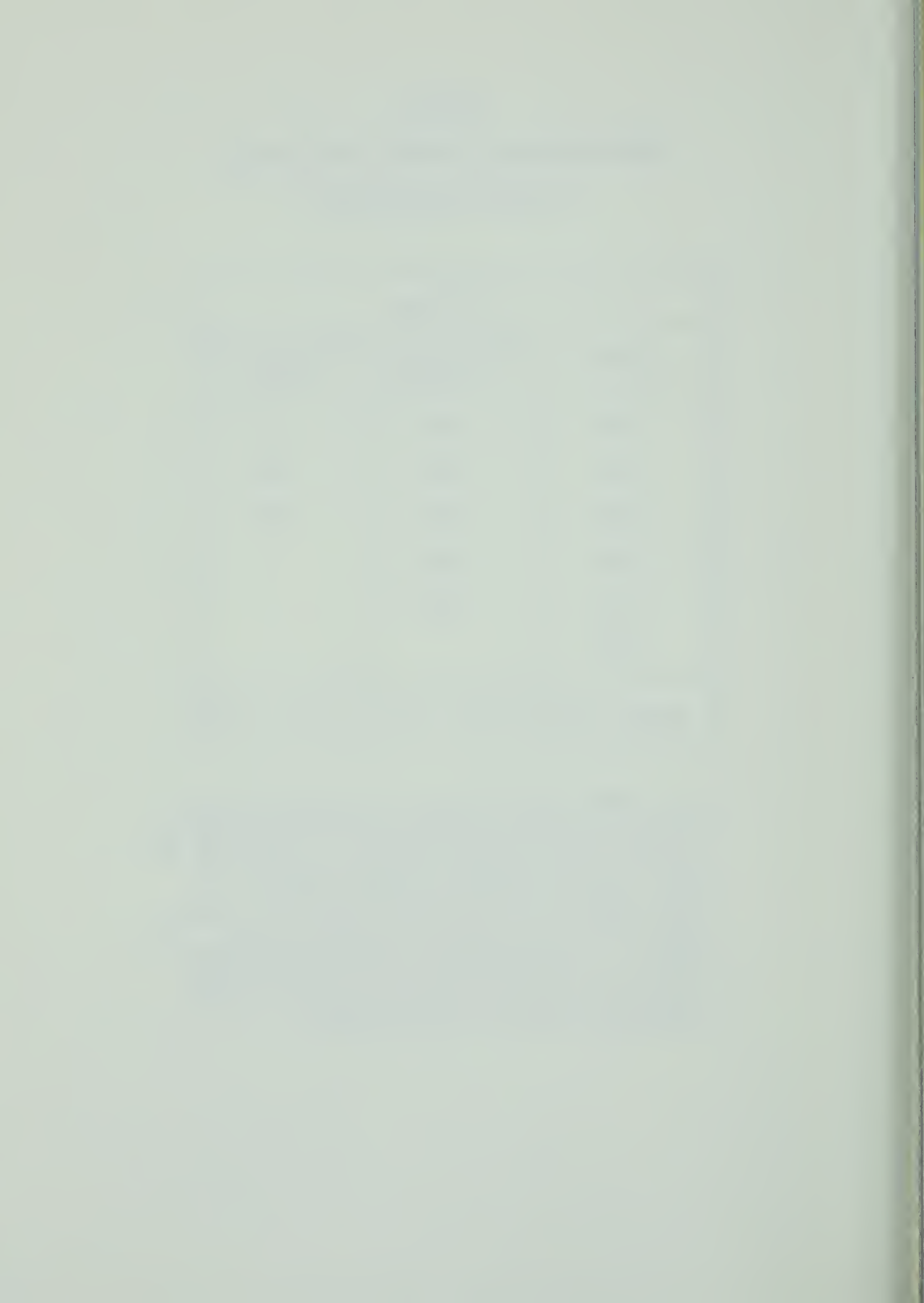


Table 5

Magnesium Content in EDTA-Treated F-Actin  
or Chelex-Treated Myosin

-Δ O.D. <sub>640</sub>		
Control	EDTA-treated F-actin	Chelex-treated myosin
0.125	0.116	0.137
0.113	0.165	0.123
0.113	0.108	0.141
0.126	0.107	
0.110	0.132	
0.162		
0.124 ± 0.006	0.126 ± 0.024	0.134 ± 0.009

Myosin (~500 mg) was treated by passing through a 2.5 x 15 cm column of chelex pre-equilibrated with 0.5M KCl, 0.02M histidine (pH 7.4). F-actin (39 mg in 100 ml) was treated by dialysing against 1mM EDTA, 0.1M KCl overnight, and then against 5 x 1 litre changes of 0.1M KCl containing 100 ml chelex. Samples (3.1 mg F-actin, 27.3 mg myosin) were tested. Any contamination would be indicated as a difference in calmagite absorption between sample and control means, the control being TCA-treated dialysate or filtrate of the proteins.



and an appropriate amount of carrier, was filtered through a Millipore filter (100  $\mu$  pore size). Not more than 5% by volume of the reaction mixture was filtered for assay so that the protein concentration was not altered appreciably. Aliquots (0.2 ml each) from the reaction mixture and the filtrate were added to scintillation vials, and 0.8 ml of NCS reagent (Nuclear Chicago Corp., Des Plaines, Ill., U.S.A.) was added to solubilize the protein. Ten ml of fluor containing 5.5 g of Pre-mix 'M' (Packard Instrument Co., La Grange, Ill., U.S.A.) was added to each vial. Counting was done in a Beckman LS-100 scintillation counter using the variable isoset. External standardization with  $^{137}\text{Cs}$  showed no quenching. A counting efficiency of 83% was not changed in a concentration range of  $\text{CaCl}_2$  up to 0.1mM (same volume as for samples). At the lowest concentration of  $\text{CaCl}_2$  used in these experiments 0.1 ml of a solution without protein gave  $1900 \pm 19$  counts/min before filtration and  $1817 \pm 18$  counts/min after filtration, the difference of 4.4% suggesting some binding of  $\text{Ca}^{2+}$  to the membrane. The radioactivity of the reaction mixture and the filtrate, representing the total and unbound  $\text{Ca}^{2+}$  concentrations, respectively, showed a difference of not less than 50% up to a concentration of  $5.0 \times 10^{-7}\text{M}$  added  $\text{CaCl}_2$ , meaning that in this range, the error due to membrane binding would not exceed 10%.

#### C. Magnesium binding to actin and myosin

A technique for determining micromolar concentrations of  $\text{Mg}^{2+}$  spectrophotometrically using an azo dye, calmagite, was developed. This dye is very soluble and stable in aqueous solutions, unlike eriochrome black T (Lindstrom and Diehl, 1960) which had been used



previously (Kuby et al., 1962; Cantor and Hearst, 1969), and is more sensitive than other dyes (Chauhan and Sarkar, 1969). Both  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$  react to form a 1:1 complex with calmagite resulting in a color change from blue to red. The absorption spectra of 0.1mM calmagite in  $\text{NH}_4\text{OH}-\text{NH}_4\text{Cl}$  ( $\mu=0.4$ , pH 10) and the  $\text{Mg}^{2+}$  complex of calmagite (Fig. 3) compare favourably with that of Lindstrom and Diehl (1960). The colour change is complete within 15 minutes and remains stable for 4 hours (Chauhan and Sarkar, 1969). It is not expected that the reaction of transition metals (Chauhan and Sarkar, 1969) would present a problem unless there is a high level of contamination in the protein.

The dye was made in a 10mM aqueous solution and was stored in the dark in a polyethylene bottle (Lindstrom and Diehl, 1960). An aliquot of this stock solution was used to prepare a dilute solution daily (0.1mM calmagite,  $\text{NH}_4\text{OH}-\text{NH}_4\text{Cl}$ ,  $\mu=4$ , pH 10). The standard curve was constructed by adding the dilute calmagite solution to the samples of  $\text{MgCl}_2$  (1-10mM in 0.01M KCl, 0.01M histidine, pH 7.4) which had been evaporated to dryness at  $75^\circ\text{C}$  in a vacuum oven. The addition of the dye solution to dry samples caused no change in the dye concentration, and the change in absorbance was measured very accurately by the double beam mode in a Cary 16 spectrophotometer. Since there is a decrease in absorbance upon addition of  $\text{Mg}^{2+}$ , the position of the reference and the sample are reversed and the readings were recorded as  $-\Delta \text{O.D. } 640$ . Successful determinations have been also made at 610, 615, and 620 m $\mu$ , but with less sensitivity. The experiments shown in Fig. 4 were designed to illustrate the main characteristics of this determination. At the concentration of calmagite used, the  $-\Delta \text{O.D.}$  at 615 or 640 m $\mu$  was essentially linear up to 10 $\mu\text{M}$   $\text{MgCl}_2$ . A certain amount of histidine and/or KCl appeared to be







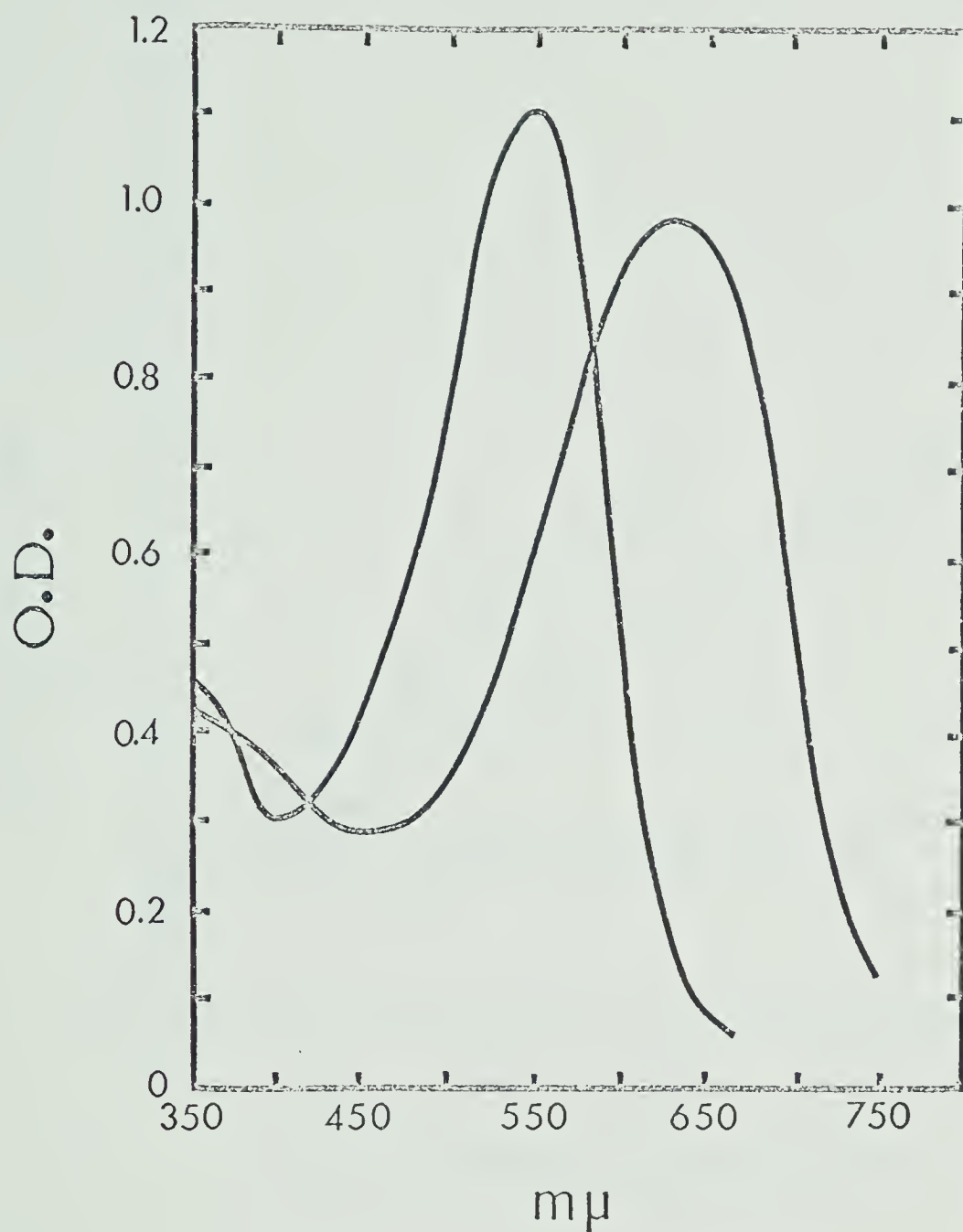


Figure 3. Absorption spectra of calmagite. Calmagite (0.1mM in  $\text{NH}_4\text{OH} - \text{NH}_4\text{Cl}$ ,  $\mu = 0.4$ , pH 10) was tested in a 1 cm cuvette of a Beckman DU-2 spectrophotometer (right-hand peak). The addition of an excess of  $\text{MgCl}_2$  (1.0mM) shifted the peak to the left. The maximum absorbance difference occurs at 610 - 640 mμ.



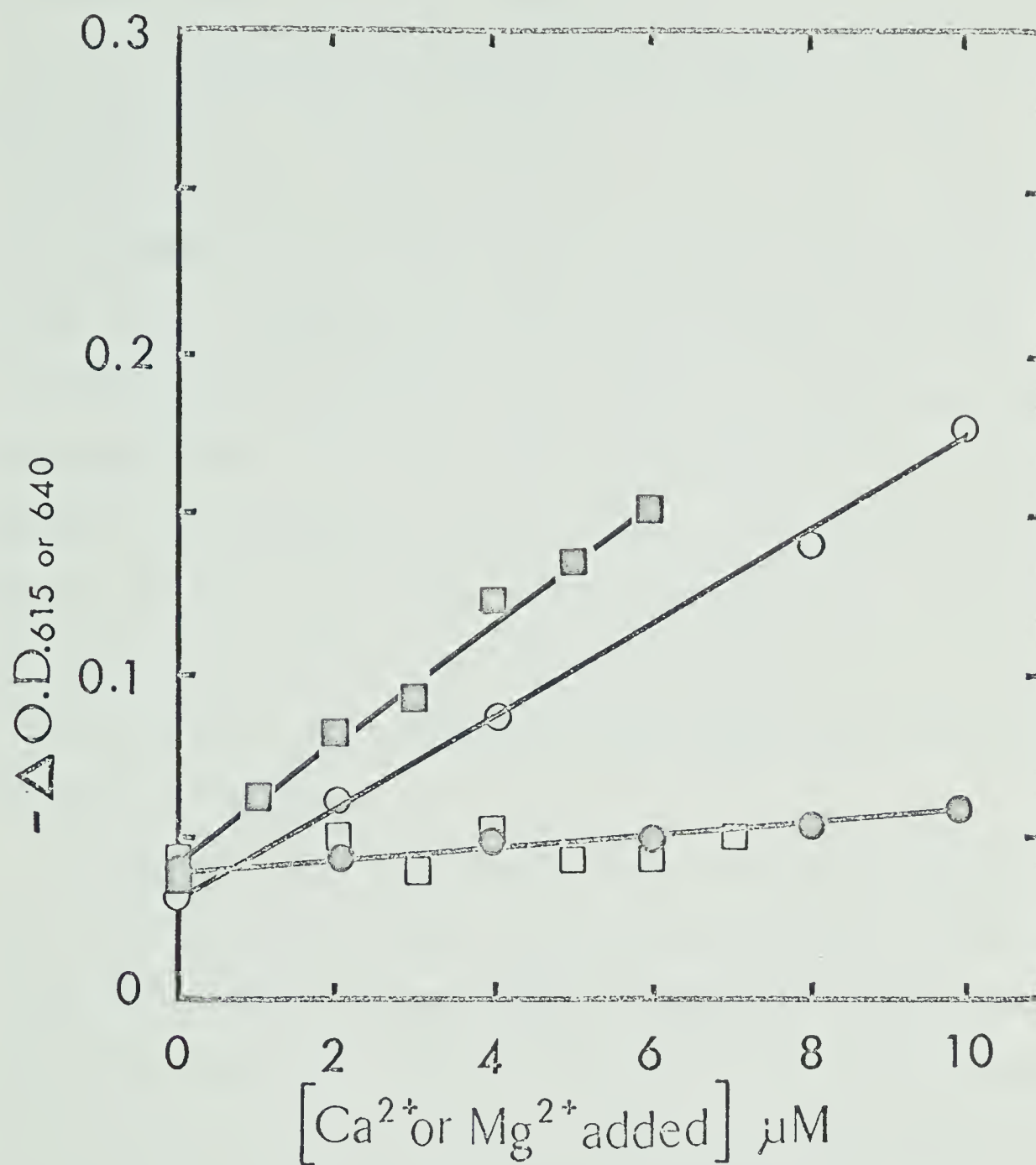


Figure 4: Absorbance changes of calmagite by the addition of Ca<sup>2+</sup> and Mg<sup>2+</sup>. Calmagite (0.1mM in NH<sub>4</sub>OH - NH<sub>4</sub>Cl,  $\mu$  = 0.4, pH 10) was used. (○), MgCl<sub>2</sub> in 0.01M KCl, 0.01M histidine; (●), CaCl<sub>2</sub> in 0.01M KCl, 0.01M histidine; (□), MgCl<sub>2</sub> in <0.001M KCl, <0.001M histidine. The above determinations were made at 615 mμ. (■), MgCl<sub>2</sub> in 0.01M KCl, 0.01M histidine, 640 mμ.



necessary, otherwise the  $Mg^{2+}$  did not react with calmagite, probably because of absorption by glass after the sample was dried. Compared with  $Mg^{2+}$ ,  $Ca^{2+}$  did not react to any extent (see also Lindstrom and Diehl, 1960; Chauhan and Sarkar, 1969). The change in absorbance detected without added magnesium did not appear to be due to any contamination in KCl and/or histidine, since no correlation could be observed with varied concentrations of these substances (Table 6). There was no change in pH of the reaction mixture. This unexplained solvent effect is even greater with tris buffer, and due to some degree of fluctuation imposes a technical limitation on this test. In a given set of determinations, however, the solvent effect was consistent between samples and good standard curves were obtained.

In summary, by using the stable, highly sensitive calmagite, and by adding this to a dried sample so as to minimize variations in the concentration of dye, micromolar quantities of  $Mg^{2+}$  were determined accurately. This is an improvement of recent, similar, techniques using eriochrome black T (Cantor and Hearst, 1969) or calmagite (Chauhan and Sarkar, 1969).

All glassware was washed with dilute nitric acid and 5mM EDTA (pH 10), followed by a thorough rinse with double distilled water. Myosin in 0.5M KCl, 0.02M histidine (pH 7.4) was dialysed versus a large volume of chelex (100 ml). It was further dialysed to bring the final concentration to 0.01M KCl, 0.01M histidine (pH 7.4). At 5-10 mg/ml, the myosin-gel could be quantitatively pipetted upon rinsing the pipet with the resulting solution.



Table 6

Solvent Effect on Calmagite Absorption

histidine mM	KCl mM	Calmagite mM	$-\Delta$ O.D. <sub>610</sub>	$-\Delta$ O.D. <sub>640</sub>
0.9	<1	0.01	0.0164	
10	12	0.01	0.0985	
10	12	0.01	0.0301	
10	100	0.02	0.0262	
0.6	<1	0.10	0.0336	
10	10	0.10	0.0694	
10	10	0.10		0.0250
10	12	0.10		0.0354
10	17	0.10	0.0970	
10	100	0.10		0.0842

The solvent is the filtrate from the  $Mg^{2+}$  binding studies where the original solution contained no added  $Mg^{2+}$  or protein. These were dried and analysed in the same way as samples. There was no change in absorbance when KCl and histidine were absent.





The  $\text{Mg}^{2+}$  binding reaction was determined in an Amicon Model 12 10 ml ultrafiltration cell with a Millipore filter (100 mμ pore size). All parts of the filtration apparatus were soaked overnight in EDTA. After assembly, the cell was rinsed thoroughly with 5 changes of distilled water and a liter of dialysate (0.01M KCl, 0.01M histidine, pH 7.4). The filtration cell contained 10 ml of the reaction mixture. With constant stirring to prevent concentration at the cell base, no more than 20% filtrate was expressed using 50 psi of nitrogen (ultrapure, Linde). This pressure is too low to affect the ATPase or the actin-myosin association reaction (Laidler, 1951; Ikkai and Ooi, 1969). In a given run, standards were also filtered. Aliquots (1 ml each) of samples and standards were dried at 75°C in 20 ml beakers under a reduced pressure. Then 1 ml of 0.1mM calmagite in  $\text{NH}_4\text{OH-NH}_4\text{Cl}$  ( $\mu=0.4$ , pH 10) was added. After 15 minutes, the  $-\Delta \text{O.D. } 640$  was determined as described.

#### D. Calcium and magnesium binding to adenosine and inosine triphosphates

A technique was employed in which the spectral change of a dye, 8-hydroxyquinoline, was analysed to determine the association constants of  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$  to ATP or ITP (Burton, 1959) under the conditions of the enzymatic assays. A concentration of 0.8mM and 0.3mM 8-hydroxyquinoline was used for  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$  binding, respectively. Burton considered the reaction of the divalent cations, M, with the nucleoside triphosphate, P, in three possible complexes: MP,  $\text{M}_2\text{P}$ , and  $\text{MP}_2$ . Furthermore he considered the possible formation of a ternary complex between the cation, nucleoside triphosphate, and the dye. He showed that the association constant K, of MP is given by



$K = 1/\{M\}_{1/2}$  where  $\{M\}_{1/2}$  is the free cation concentration when  $\{MP\} = \frac{1}{2}\{Po\}$ ,  $\{Po\}$  representing the total concentration of the nucleoside triphosphate. However, the apparent free concentration of  $Mg^{2+}$ ,  $\{M\}_{1/2}'$ , was found by Burton to depend on the concentration of  $\{Po\}$ . Thus  $K$  is represented by

$$K = \frac{1}{\{M\}_{1/2}} + k\{Po\}$$

where  $k$  is a factor introduced by Burton to account for this dependence, attributed to the formation of a ternary complex, and is expressed by the following equation:

$$k = \frac{\{M\}_{1/2}' - \{M\}_{1/2}}{K\{M\}_{1/2}\{P\}}$$

where  $\{P\}$  is the concentration of uncombined nucleoside triphosphate. Here  $K$  is found by plotting  $\{M\}_{1/2}'$  for various values of  $\{Po\}$  and extrapolating to  $\{Po\} = 0$ . A linear plot of the data was obtained (Fig. 5) in agreement with the findings of Burton (1959) and O'Sullivan and Perrin (1964). A best fit was extrapolated and the value of  $K$  obtained from the reciprocal of the ordinate intercept (see Table 7).

## 5. Superprecipitation of Natural and Reconstituted Actomyosin

The process of superprecipitation was measured as an increase in turbidity. To a buffer solution being stirred in a 2 cm Zeiss cell was added actomyosin which raised the KCl concentration in the buffer to 0.01-0.02M. The increase in absorbance at 545 mμ, detected by a Zeiss PMQ II spectrophotometer, was recorded on a Texas



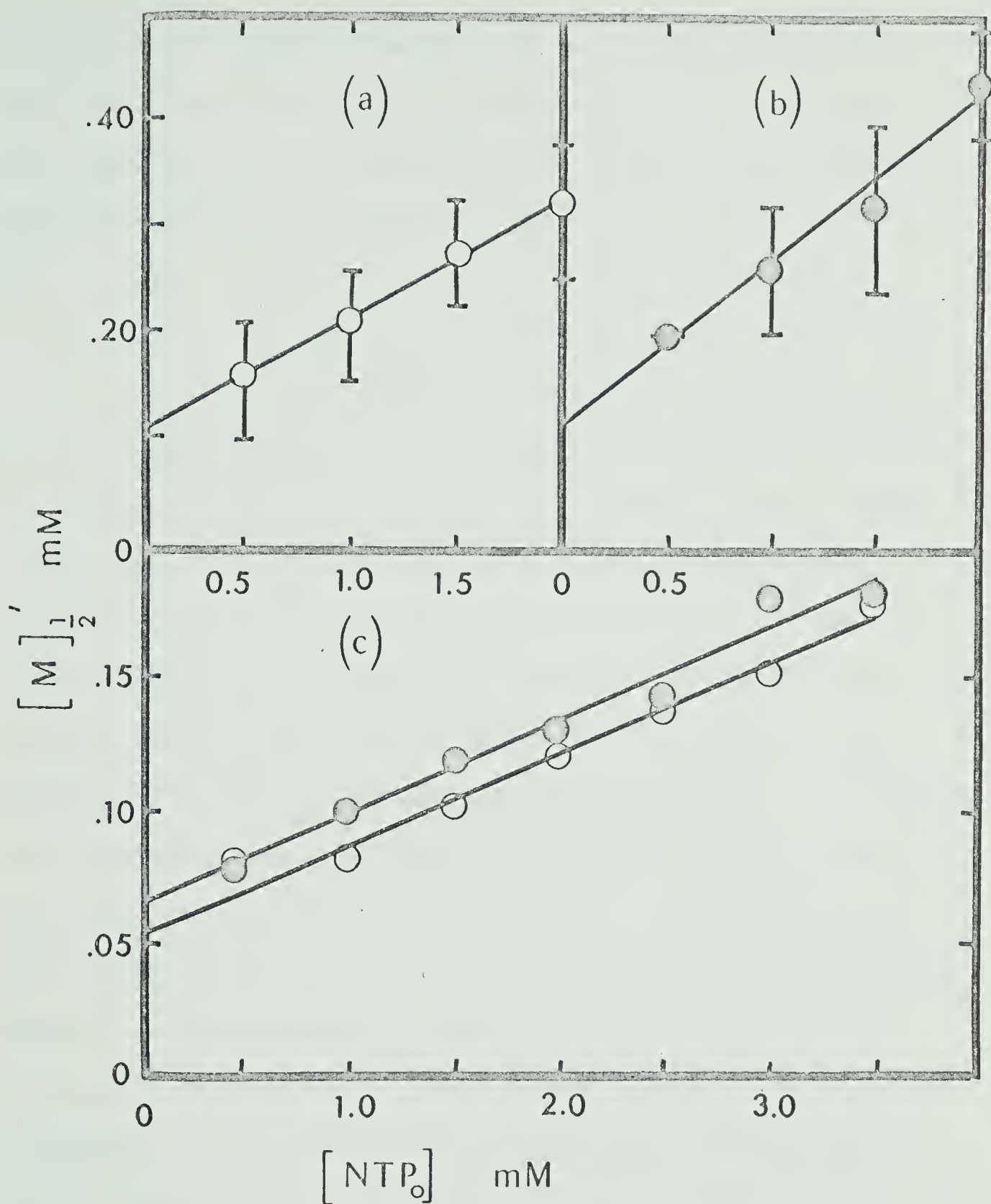


Figure 5:  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$  binding to ATP and ITP measured by the spectral change in 8-hydroxyquinoline. Conditions: 0.05M KCl, 0.05M tris-Cl (pH 7.4), 25°C, 360 mμ. (a)  $\text{CaATP}^{2-}$ , 0.8mM hydroxyquinoline; (b)  $\text{CaITP}$ , 0.8mM hydroxyquinoline; (c)  $\text{MgATP}$  (○), and  $\text{MgITP}$  (●), 0.3mM hydroxyquinoline. Vertical bars indicate standard deviation for several determinations at each concentration of NTP.



Instruments Serva-riter recorder (Levy and Fleisher, 1965; Nihei, 1967). The rate of superprecipitation was measured from the reciprocal of the time required for the turbidity change to reach 50% of the total extent (Nihei, 1967; Nihei and Yamamoto, 1969).

## 6. Light Scattering of the Actin

### Subfragment Complex

The angular distribution of polarized light (436 mμ) scattered by F-actin in the absence and presence of subfragment was examined using a Brice Phoenix Series 2000 light scattering photometer (Phoenix Precision Instrument Co., Phil., Pa., U.S.A.). Light from a high pressure mercury vapour lamp, type AH3, was either vertically or horizontally polarized by the polaroid discs. After passing through a narrow beam diaphragm and being scattered by the solutions, the intensity of scattered light was measured by a galvanometer and recorded on the Texas Serva-riter recorder. A pyrex cylindrical cell, C-101, was cemented to a square glass plate using duPont Duco cement. To test the correct positioning of the cell, a dilute solution of fluorescein was viewed through a yellow filter, and the intensity of green fluorescence was distributed equally in the angular range from 30 to 135° with 2% deviation (Zimm, 1948).

F-actin was polymerized at 3.4 mg/ml with 1mM MgCl<sub>2</sub>, 0.01mM tris-Cl (pH 7.4), which was the solvent condition used for these measurements. F-actin was dialysed to remove ATP. After dialysis, F-actin was diluted with the solvent which had been twice filtered through a Millipore filter (250 mμ pore size). The solutions were







centrifuged in a Sorvall HB-4 swinging bucket rotor at 10,000 rev./min. for 20 minutes to remove dust (Oosawa et al., 1959). The protein concentration did not change after centrifugation. After standing 1 hour at room temperature to achieve thermal equilibration (Oosawa et al., 1959), 20 ml of F-actin suspension was transferred to the C-101 pyrex cylindrical cell. The myosin subfragment, at 4.7 mg/ml in 0.05M KCl, 0.01M  $K_2HPO_4$  (pH 7.4), 1mM D.T.T. was centrifuged at 100,000 x g for 2-3 hours before adding to solutions of F-actin. The data was corrected according to the equation of Tomimatsu and Palmer (1963) which accounts for extra light arising from back reflection.

## 7. Sedimentation-Velocity and High-Speed Sedimentation-

### Equilibrium Analysis of Myosin Subfragment

Sedimentation-velocity studies of subfragment were carried out in a Spinco Model E analytical ultracentrifuge at 59,780 rev./min. with rotor temperature maintained at 20°C.

The molecular weight and homogeneity of subfragment were tested using the high-speed sedimentation-equilibrium technique of Yphantis (1964). This technique does not require a separate estimation of protein concentration and is useful for detecting pauci-dispersion. Rayleigh interference optics were used for the measurement of solute distribution in a centrifugal cell. A sample solution (0.11 ml) and its dialysate (0.12 ml) were placed in a 12mm, double-sector cell with a Epon-filled centerpiece. The counterbalance of the An-D rotor was equipped with inner and outer reference holes, the former having a wire for more precise measurements. The phase bar



angle was  $90^{\circ}$ . Photographs were taken with Kodak type 11-G high contrast spectral plates and the fringe displacements were measured with a Nikon comparator. A density of 1.025 at  $4^{\circ}\text{C}$  was calculated from the contribution of individual solution components (0.5M KCl, 0.01M  $\text{K}_2\text{HPO}_4$ , pH 7.4, 0.02-0.1M  $\beta$ -mercaptoethanol).

A plot of the fringe displacement as a function of the distance from the meniscus indicated zero protein concentration near the meniscus, a necessary condition for the Yphantis technique (Fig. 6).

## 8. Chemicals

ATP and ITP were purchased from the Sigma Chemical Co., and used without further treatment except when chelex treatment was required. Calcein and calmagite were purchased from the G.F. Smith Chemical Co., Columbus, Ohio, U.S.A. Other chemicals were of reagent grade. The stock solutions of  $\text{CaCl}_2$  and  $\text{MgCl}_2$  were standardized by titration against EDTA.

## III. RESULTS

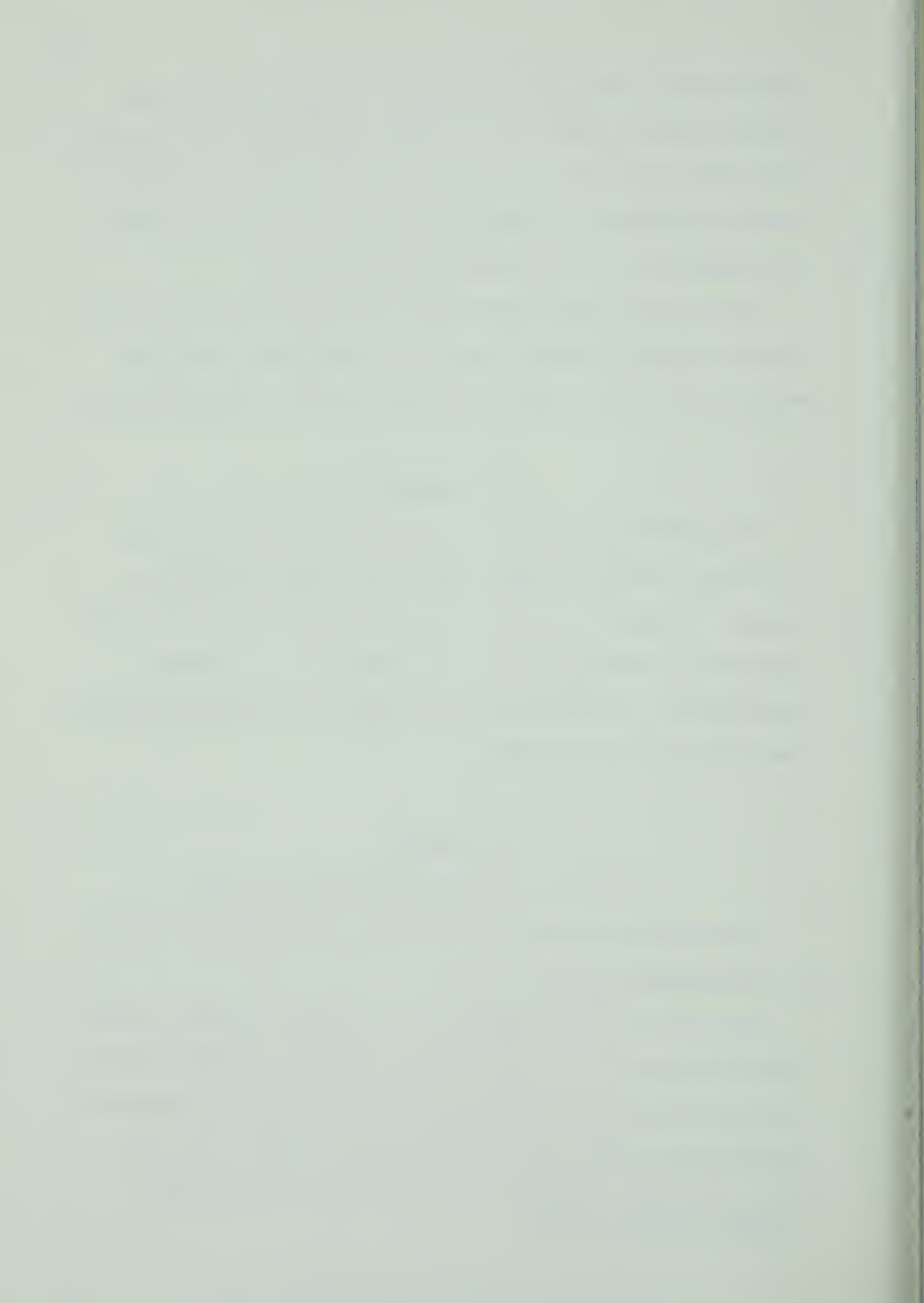
### 1. The Effects of Calcium and Magnesium on the

Adenosine Triphosphatase and Inosine Triphosphatase of Myosin\*

#### A. Activation by Calcium

The effects of  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$  on the enzymatic activity of myosin were studied at a KCl concentration of 0.05M, the enzymatic activity of myosin at this concentration not being affected by a variation of monovalent ion concentrations in the range of  $\pm 0.03\text{M}$ . Also, the

\*Most of the work described in this section has been published (Sugden and Nihei, 1969).



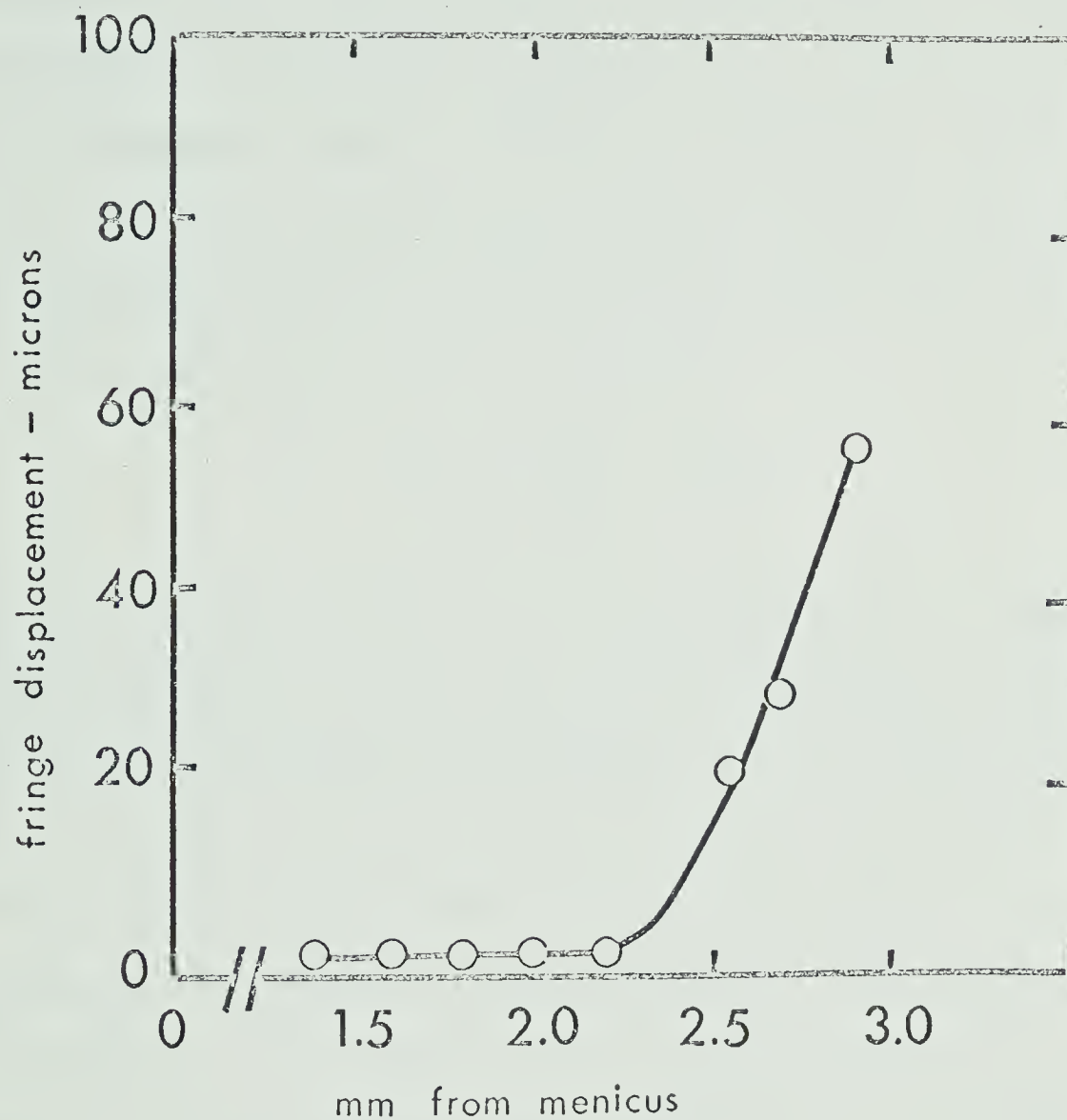
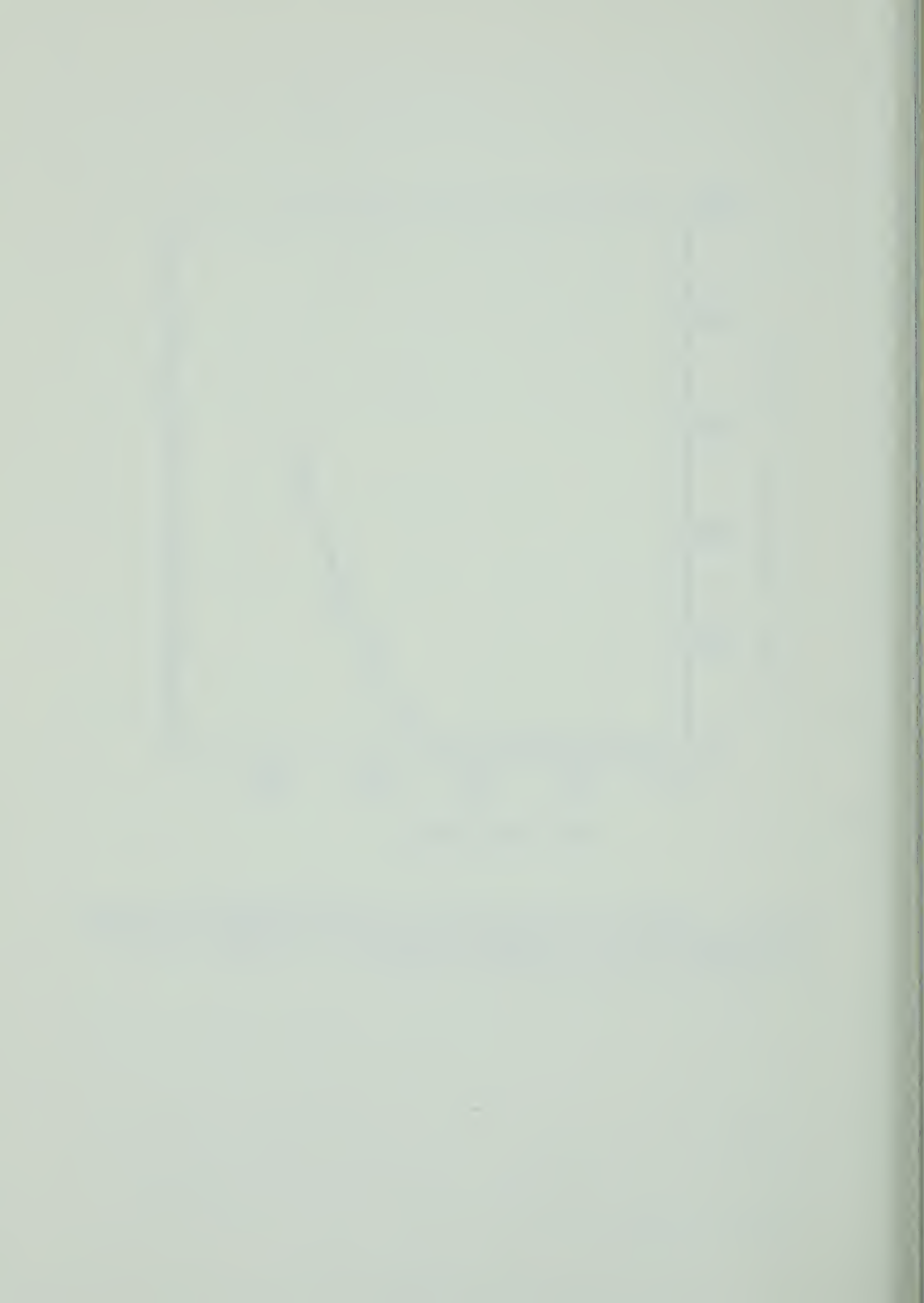


Figure 6. Sedimentation-equilibrium of myosin subfragment (0.5M KCl, 0.01M  $K_2HPO_4$ , pH 7.4, 0.1M  $\beta$ -mercaptoethanol, 1.0 mg/ml, 4°C). Run duration was 24 hours at 18,000 rev./min.



characteristics of myosin ATPase in 0.05M KCl could be compared with those of actomyosin ATPase associated with superprecipitation. As shown in Fig. 7a and 7b,  $\text{Ca}^{2+}$  at concentrations up to 30mM activates the hydrolysis of ATP and ITP, and further increase in its concentration makes the  $\text{Ca}^{2+}$  effect inhibitory. For the analysis of  $\text{Ca}^{2+}$  activation of myosin ATPase or ITPase, the rates of hydrolysis at various concentrations of  $\text{Ca}^{2+}$  may be expressed as the "relative activity",  $v$ , which is:

$$v = \frac{(v - v_1)}{(v_2 - v_1)} \times 100$$

where  $v_1$ ,  $v_2$  and  $v$  denote the rates determined with no added  $\text{Ca}^{2+}$ , with the optimum amount of added  $\text{Ca}^{2+}$  to give maximal activation, and with an intermediate concentration of  $\text{Ca}^{2+}$ , respectively. This ratio was found to be useful for the analysis of the  $\text{Mg}^{2+}$  activation of myosin B ATPase (Nihei et al., 1966). Then, to examine the possibility of a binding reaction between  $\text{Ca}^{2+}$  and myosin being responsible for the activation, the relative activity was plotted against the concentration of the free  $\text{Ca}^{2+}$ . The free  $\text{Ca}^{2+}$  was defined as the  $\text{Ca}^{2+}$  not bound to NTP (ATP or ITP). To calculate the free  $\text{Ca}^{2+}$  concentration, it was necessary to determine the apparent association constant,  $K_{app}$ , for  $\text{CaNTP}^{2-}$  under the experimental conditions. The spectrophotometric method of Burton (1959) was applied (Methods and Materials), and the apparent association constants listed in Table 7 were within the range of values reported by Nanninga (1961), Asai and Morales (1962), and O'Sullivan and Perrin (1964). The relative activity plotted against the  $\text{Ca}^{2+}$  concentration





Figure 7. ATPase and ITPase activities as a function of the added concentration of  $\text{Ca}^{2+}$ . (a); (○) 0.1mM ATP; (●), 1.0mM ATP. pMB-treated ATPase: (△), 0.1mM ATP; (▲), 1.0mM ATP. ITPase: (b); (□), 0.1mM ITP; (■), 1.0mM ITP. Conditions: 0.05M KCl, 0.05M tris-Cl (pH 7.4), 0.12 mg myosin/ ml in all cases, 25°C.



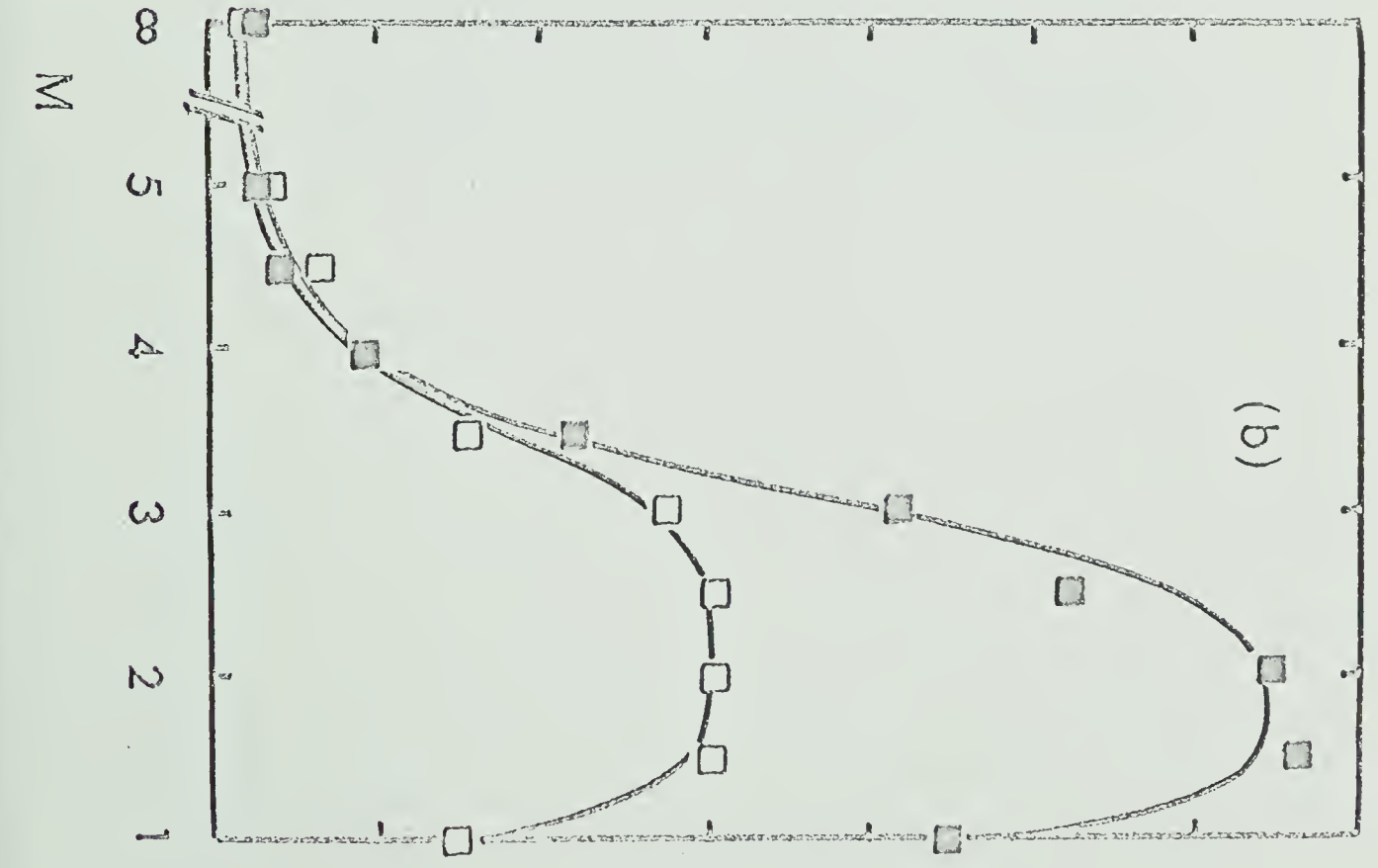
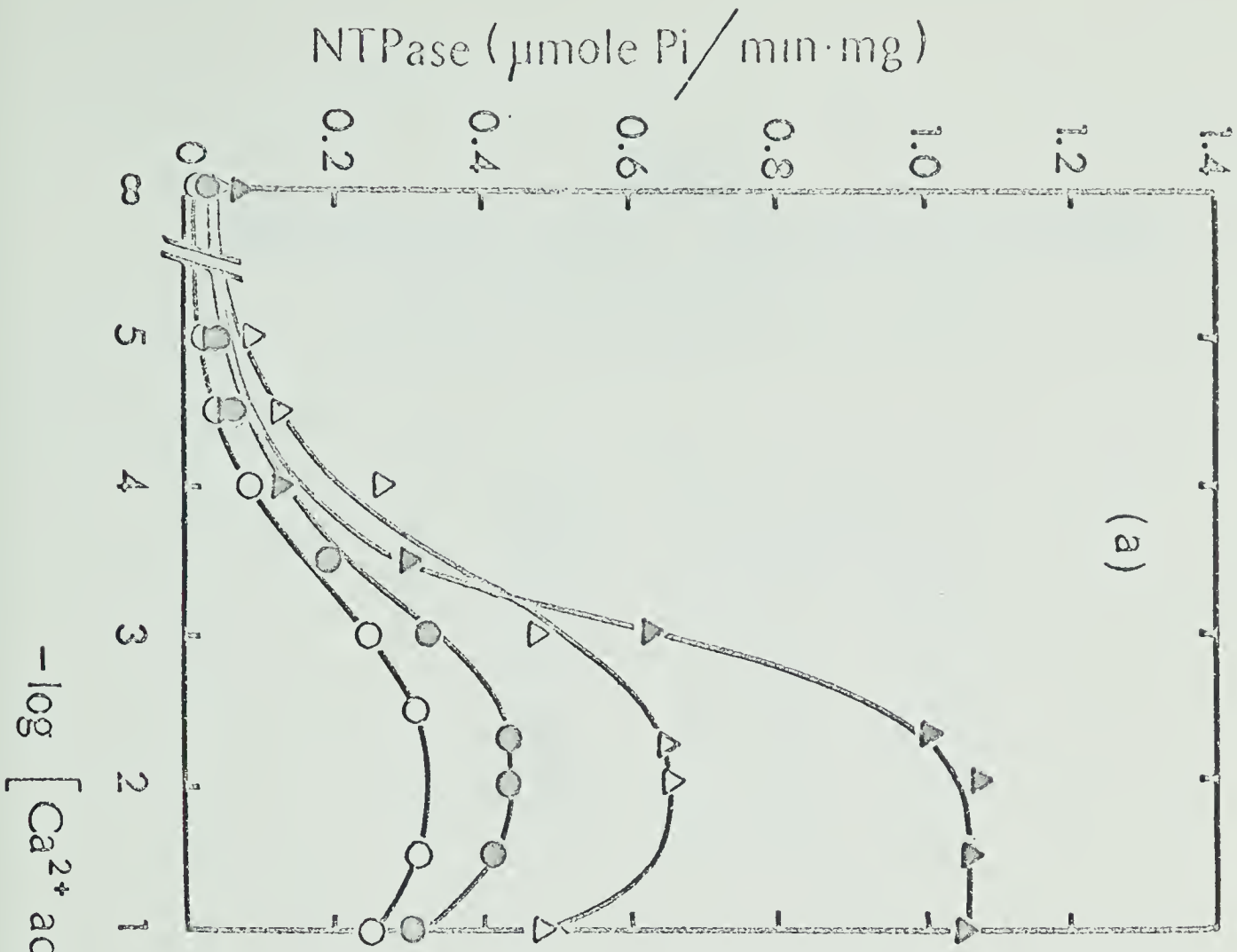




Table 7

Kapp for the  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$  Complexes with ATP and ITP  
as Determined Spectrophotometrically

Complex	Kapp ( $\text{M}^{-1}$ )
$\text{CaATP}^{2-}$	$9,300 \pm 1,400$
$\text{CaITP}^{2-}$	$8,800 \pm 600$
$\text{MgATP}^{2-}$	$18,900 \pm 800$
$\text{Mg ITP}^{2-}$	$15,400 \pm 300$

Determinations were carried out in 0.05M KCl, 0.05M tris-Cl (pH 7.4), at 25°C. A best fit of the experimental plot (Fig. 3) gave Kapp as the reciprocal of the ordinate intercept (see Methods and Materials).



(Fig. 8) suggests, from the shape of the curve, that a certain  $\text{Ca}^{2+}$  binding reaction is responsible for the activation. The binding of  $\text{Ca}^{2+}$  to myosin was therefore studied by a procedure described in Methods and Materials in an attempt to determine whether this was the reaction responsible for activation.

The binding reaction was observed to be hindered at high concentrations of myosin (Table 8). Although there was no detectable difference in the binding measured after 5 and 25 minutes incubation, it is conceivable that the lower binding observed at 2 mg/ml may be due to the anomalous effects seen with high protein concentrations in equilibrium dialysis (Cassel et al., 1969). The precipitated myosin suspension could provide the same barrier to equilibrium approach as does the coating of dialysis membranes with protein. Therefore a concentration of 0.67 mg/ml of myosin was used. The resultant Scatchard (1949) plot suggests that there are at least two types of binding reactions between  $\text{Ca}^{2+}$  and myosin (Fig. 9). The first type is a binding reaction that occurs at a site present on each 500,000 atomic mass units of myosin and has a  $K_{app}$  of  $1.3 \times 10^6 \text{ M}^{-1}$ . The second type is a weak binding, the binding parameters of which cannot be accurately measured due to the low protein concentration. The results obtained in the presence of ATP show a binding similar to that in the absence of ATP. In this case the plot is made ignoring the  $\text{CaATP}^{2-}$  formation, which is insignificant within the range of  $\text{Ca}^{2+}$  concentrations used. When myosin is modified with NEM, which is equivalent to PMB in its effect on the ATPase activity (Kielley and Bradley, 1956), the tight  $\text{Ca}^{2+}$





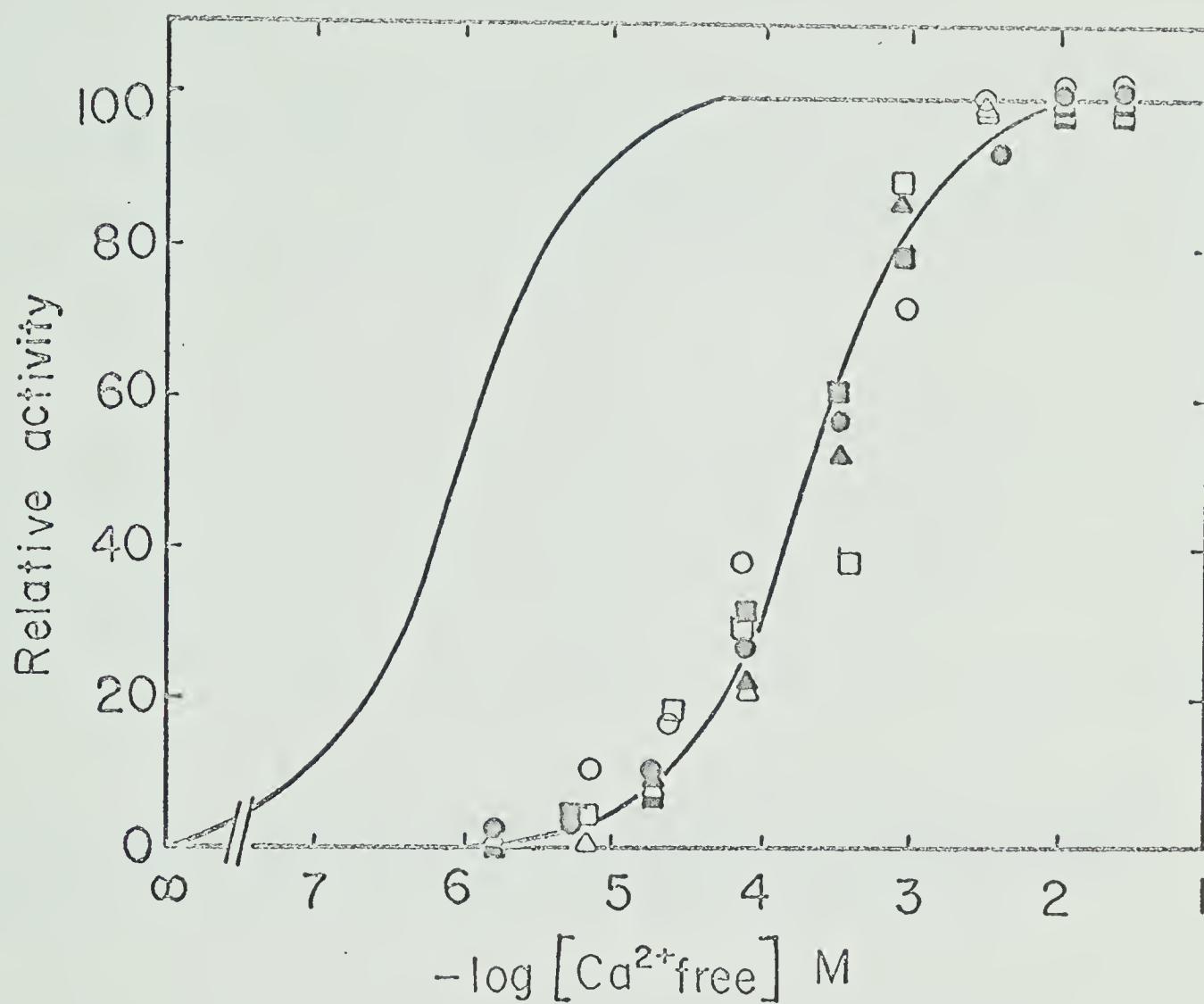


Figure 8: Relative activity as a function of the calculated concentration of free  $\text{Ca}^{2+}$ . Same conditions and symbols as for Figure 6. The continuous curve to the left depicts the tight  $\text{Ca}^{2+}$  binding to myosin.



Table 8

Effect of Protein Concentration on the  
Binding of Ca<sup>2+</sup> to Myosin

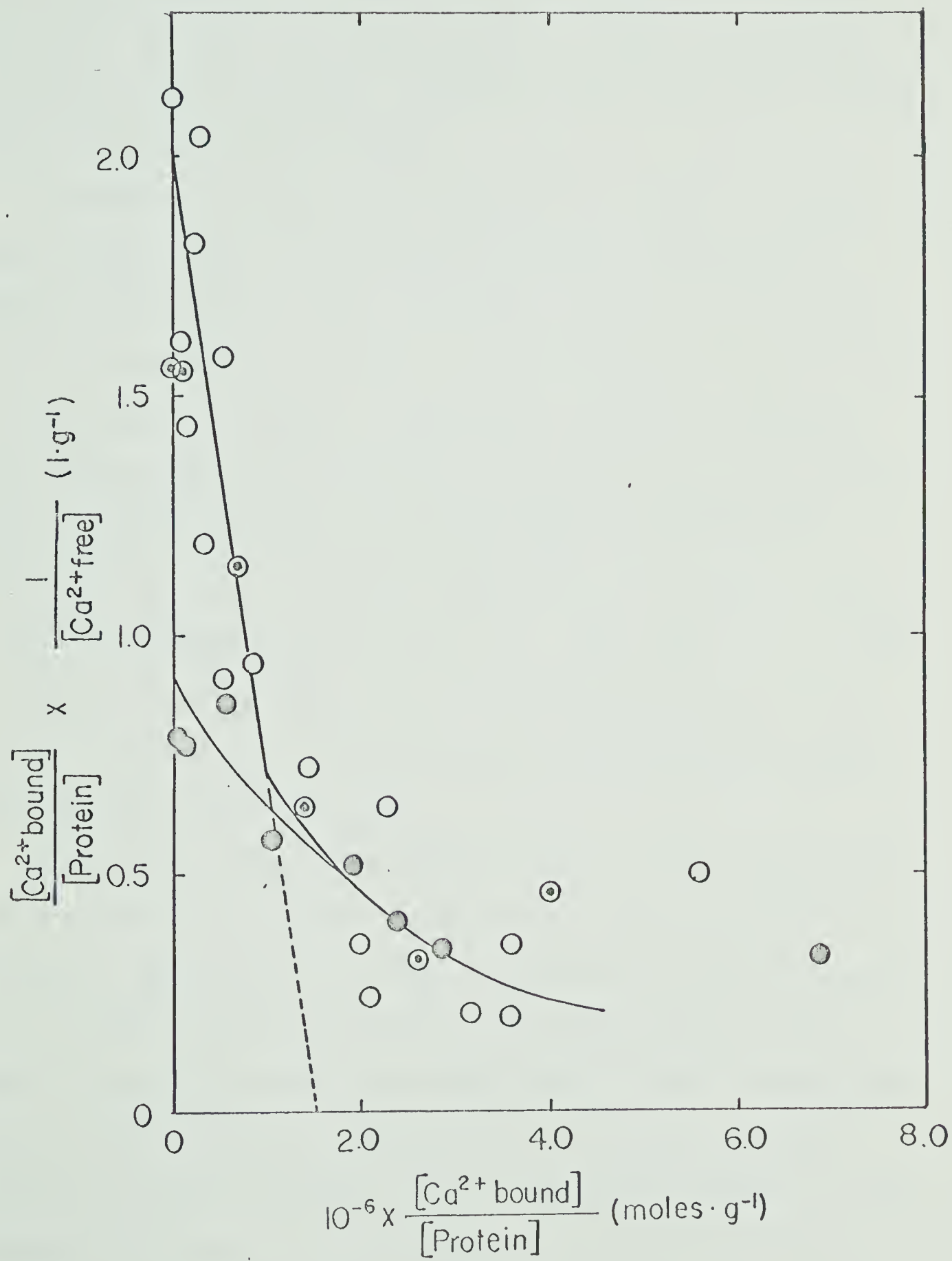
Conc. of protein (mg/ml)	Time of mixing (minutes)	$\frac{\{Ca^{2+} \text{ bound}\}}{\{Protein\}} \times \frac{1}{\{Ca^{2+} \text{ free}\}} (l.g^{-1})$
0.15	5	1.9
0.33	5	2.1
0.67	5	2.1
2.12	25	0.7
2.05	5	0.7

A fixed amount of Ca<sup>2+</sup> (0.3 nmole) was added to each 10 ml of myosin in 0.05M KCl, 0.05M tris-Cl (pH 7.4), at 25°C. The assay of Ca<sup>2+</sup> bound was performed as described in Methods and Materials.



Figure 9. Scatchard plot of  $\text{Ca}^{2+}$ -binding by native and NEM-modified myosin. Myosin was modified with 2.0 moles of NEM/ $10^5$  g. of myosin for 30 minutes and the reaction was stopped with a 50-fold molar excess of  $\beta$ -mercaptoethanol. ( $\bigcirc$ ), unmodified myosin, no ATP; ( $\odot$ ), unmodified myosin, 1.0mM ATP; ( $\bullet$ ), NEM-modified myosin, no ATP. Conditions: 0.05M KCl, 0.05M tris-Cl (pH 7.4), 25°C.









binding is weakened (Fig. 9). This is in agreement with the observation by Kitagawa et al. (1961) that myosin released the tightly bound  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$  when treated with pMB. Despite its decreased capacity to bind  $\text{Ca}^{2+}$  tightly, the NEM or pMB-treated myosin retains the  $\text{Ca}^{2+}$  activated myosin NTPase.

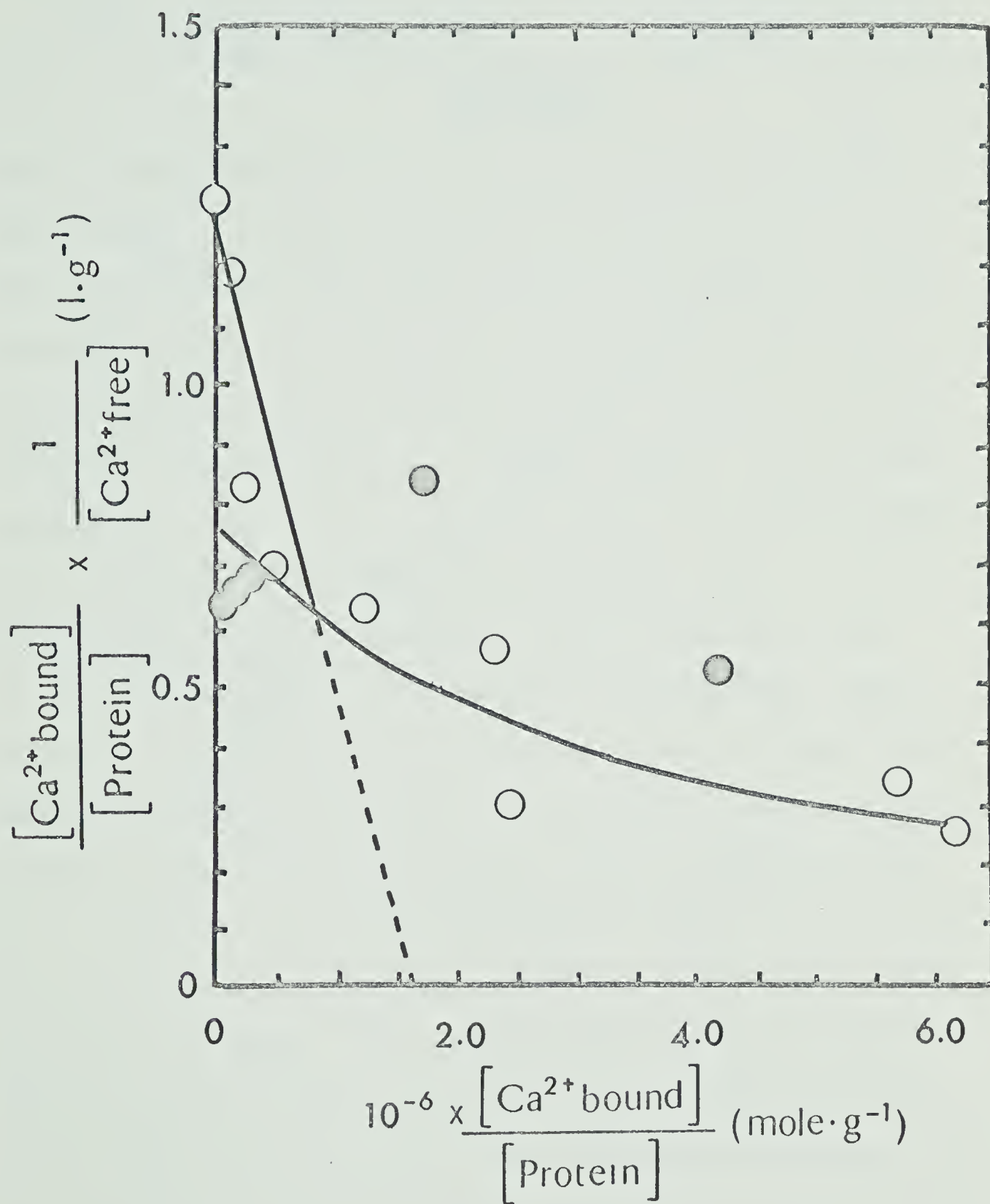
Although the presence of AMP deaminase, myokinase, or nucleic acid in myosin does not significantly alter the protein mass or ATPase characteristics (Byrnes and Suelter, 1965), it is also important to establish that this contamination does not alter the  $\text{Ca}^{2+}$  binding reaction to myosin. A combination of cellulose phosphate and DEAE-cellulose chromatography removes AMP deaminase and nucleic acid contamination (Harris and Suelter, 1967; see Methods and Materials) and there is evidence that myokinase is removed by DEAE-cellulose chromatography (Richards et al., 1967; Lowey et al., 1969). A test of the  $\text{Ca}^{2+}$  binding reaction to this purified myosin shown in the Scatchard plot of Fig. 10 compares favourably with the data for unpurified myosin (Fig. 9).

Although the above results indicate that the  $\text{Ca}^{2+}$  bound tightly to myosin does not cause the activation, and that the weak  $\text{Ca}^{2+}$  binding to myosin may be the cause, it remains to be tested whether or not  $\text{CaNTP}^{2-}$  is hydrolysed faster, or at least leads to a more rapid release of products, than other species of NTP. If one assumes that the  $\text{Ca}^{2+}$  activation is due only to the formation of  $\text{CaNTP}^{2-}$ , and that the Michaelis-Menten scheme is applicable regardless of the species of the substrate, an expression of the enzymatic activity,  $v$ , at a given concentration of  $\text{Ca}^{2+}$  may be written:



Figure 10. Scatchard plot of  $\text{Ca}^{2+}$  binding by native and NEM-modified myosin purified by chromatography. Same modification and conditions as Figure 9. ( $\bigcirc$ ), unmodified myosin; ( $\bullet$ ), NEM-modified myosin. No ATP in either case.







$$\frac{v}{V} = \frac{\frac{k_3}{k_3'} \frac{\{NTP\}}{K_m' + \frac{K_m'}{K_m} \{CaNTP\} + \{NTP\}} + \frac{\{CaNTP\}}{K_m + \frac{K_m}{K_m'} \{NTP\} + \{CaNTP\}}}{\frac{\{NTP_0\}}{K_m' + \{NTP_0\}}}$$

where  $V$  is the velocity when all the substrate molecules are in the form of  $CaNTP^{2-}$ ,  $k_3$  and  $k_3'$  are the rate constants of the step at which the enzyme-substrate complex forms the products by using  $NTP$  and  $CaNTP^{2-}$  as the substrates, respectively, and  $K_m$  and  $K_m'$  are the corresponding Michaelis constants.  $\{NTP\}$ ,  $\{CaNTP\}$ , and  $\{NTP_0\}$  denote the concentrations of  $Ca^{2+}$  free  $NTP$ ,  $CaNTP^{2-}$ , and total  $NTP$ , respectively. Fig. 11 shows the plot of  $v/V$  versus  $\{CaNTP^{2-}\}/\{NTP_0\}$ . There are three curves drawn in Fig. 11, each of which is defined by the values of  $k_3/k_3'$ ,  $K_m$  and  $K_m'$ . In the special case where  $K_m = K_m'$ ,  $v/V$  becomes a linear function of  $\{CaNTP\}/\{NTP_0\}$ . Small deviations from linearity shown in Fig. 11 are due to slight differences in the Michaelis constant. The  $K_m$  values were estimated from reciprocal plots of the available data in the concentration range of 0.1-1.0mM ATP. The values so obtained are higher than determinations made at low concentrations of ATP (Kiely and Martonosi, 1968; Schiselfeld and Bárány, 1968; Lymn and Taylor, 1970), but there is little difference between the  $K_m$  values in the presence or absence of  $Ca^{2+}$  (Table 9; see also Blum, 1955; Schiselfeld and Bárány, 1968). The experimental results, therefore, illustrate that the  $Ca^{2+}$  activation is due either to a weak  $Ca^{2+}$  binding, or to a more efficient hydrolysis of  $CaNTP^{2-}$ , or to both of these.





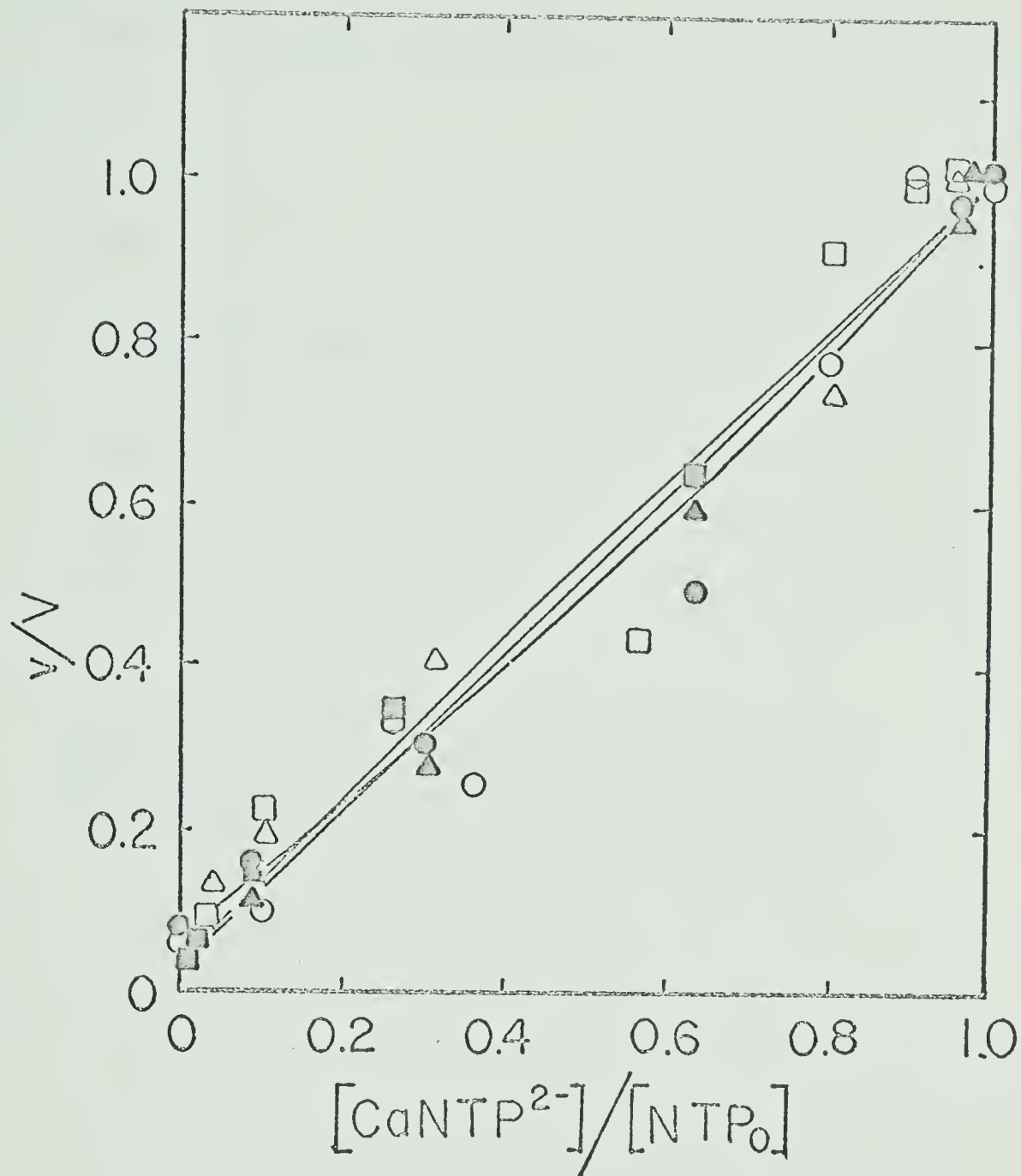


Figure 11. Ratio  $v/V$  as a function of  $\{CaNTP^{2-}\}/\{NTP_0\}$ . The theoretical curves are from the top: pMB-modified myosin ATPase, native myosin ITPase and native myosin ATPase. The symbols represent the same experimental data as shown in Figure 6.



Table 9

Michaelis Constants for Various Conditions of NTPase

	Km (M)	Km' (M)
Unmodified ATPase	$6 \times 10^{-5}$	$4 \times 10^{-5}$
Unmodified ITPase	$1.5 \times 10^{-4}$	$1.5 \times 10^{-4}$
pMB-modified ATPase	$1.1 \times 10^{-4}$	$9 \times 10^{-5}$

Km's were obtained from reciprocal plots of the kinetic data at 0.05M KCl, 0.05M tris-Cl (pH 7.4), and 25°C; Km for activities in the absence of added CaCl<sub>2</sub>, and Km' for activities at 10mM CaCl<sub>2</sub>.



## B. Inhibition by Magnesium

The ATPase activity as a function of  $\text{Mg}^{2+}$  concentration added to the reaction mixture shows that the effective concentration of  $\text{Mg}^{2+}$ , which modifies the ATPase, is lower than that of  $\text{Ca}^{2+}$ . If myosin is treated with pMB, the inhibitory effect of  $\text{Mg}^{2+}$  becomes less significant, and when 2.5 moles of pMB are used for the modification of  $10^5$  g of myosin, the inhibition by  $\text{Mg}^{2+}$  becomes unobservable (Fig. 12). Plotting the experimental results of Fig. 12 in the manner described for Fig. 7 gives two curves (Fig. 13): one for the values with 0.1mM ATP and the other for those with 1.0mM ATP. The curves appear to represent a binding reaction with  $K_{app}$  of the order of  $10^6 \text{ M}^{-1}$ , which is much larger than for the formation of  $\text{MgATP}^{2-}$ . This suggests that a binding reaction between  $\text{Mg}^{2+}$  and myosin is responsible for the inhibition by  $\text{Mg}^{2+}$ . In fact, an experiment revealed that the amount of  $\text{Ca}^{2+}$  bound to myosin decreases when  $\text{Mg}^{2+}$  is added to a mixture of myosin and  $^{45}\text{Ca}^{2+}$ . This finding is in agreement with the observed replacement of the bound  $\text{Ca}^{2+}$  from myofibrils by  $\text{Mg}^{2+}$  (Seidel and Gergely, 1963), or the lack of  $\text{Ca}^{2+}$  binding to myosin in the presence of 2.0mM  $\text{MgCl}_2$  (Fuchs and Briggs, 1968). Assuming competition between  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$  for the site on myosin, the value of  $K_{app}$  for the binding of  $\text{Mg}^{2+}$  to myosin can be estimated as  $2.1 \times 10^5 - 3.0 \times 10^5 \text{ M}^{-1}$  (Table 10). Under the experimental conditions the total  $\text{Ca}^{2+}$  concentrations are so low that the calculated amount of  $\text{Ca}^{2+}$  reacting with the weak binding sites of myosin or forming  $\text{CaATP}^{2-}$  can be assumed as negligible. With 1mM  $\text{Mg}^{2+}$ , a low value of  $K_{app}$



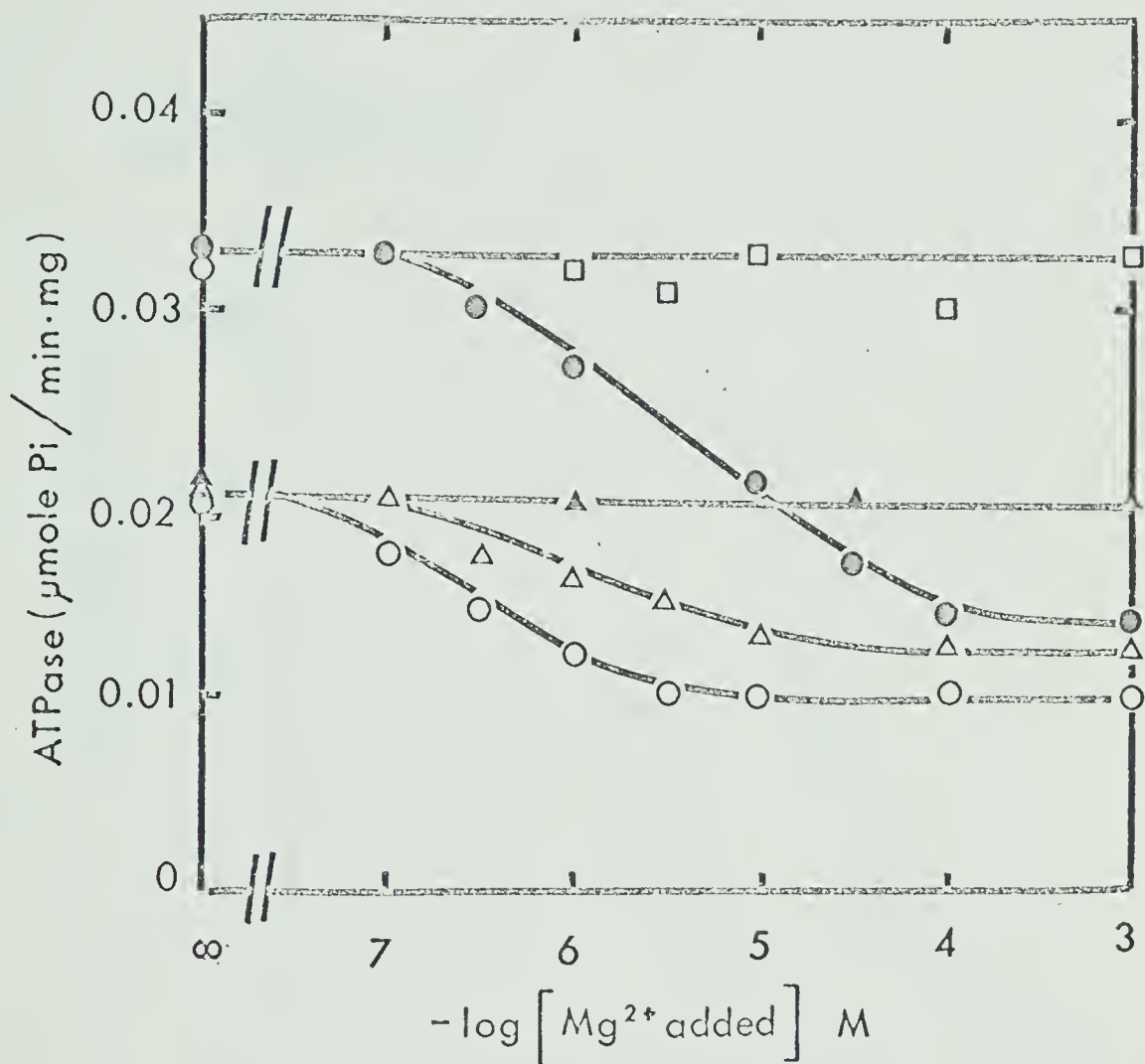
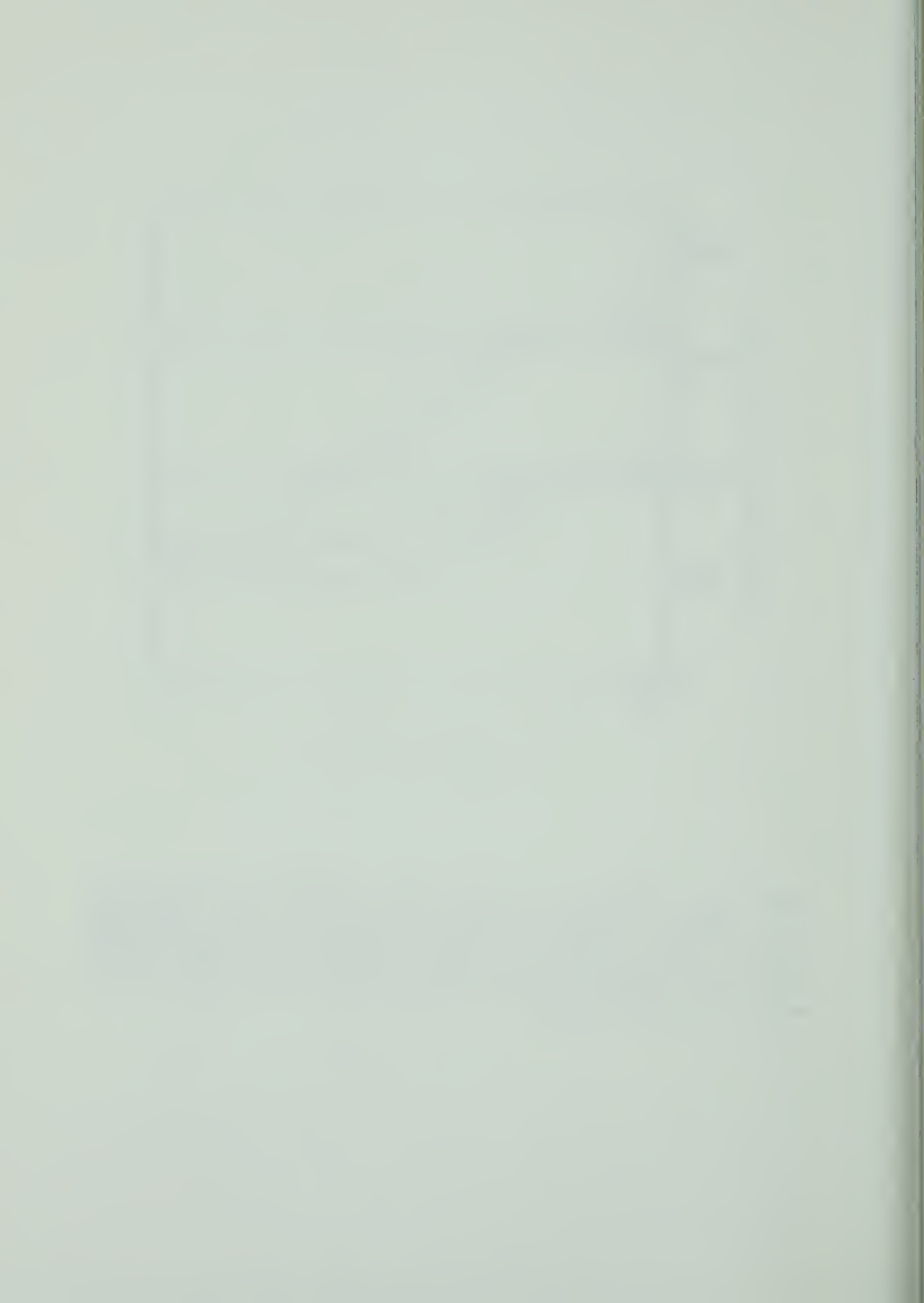


Figure 12.  $Mg^{2+}$  effect on myosin ATPase untreated or treated with pMB. ATPase: 0.05M KCl, 0.05M tris-Cl (pH 7.4), 25°C. Untreated: (○), 0.1mM ATP; (●), 1.0mM ATP. Treated myosin (1.5 moles pMB/10<sup>5</sup> g): (△), 0.1mM ATP. (2.5 moles pMB/10<sup>5</sup> g.): (△), 0.1mM ATP; (□), 1.0mM ATP. Protein concentration in all cases was 0.33 mg/ml of reaction mixture.





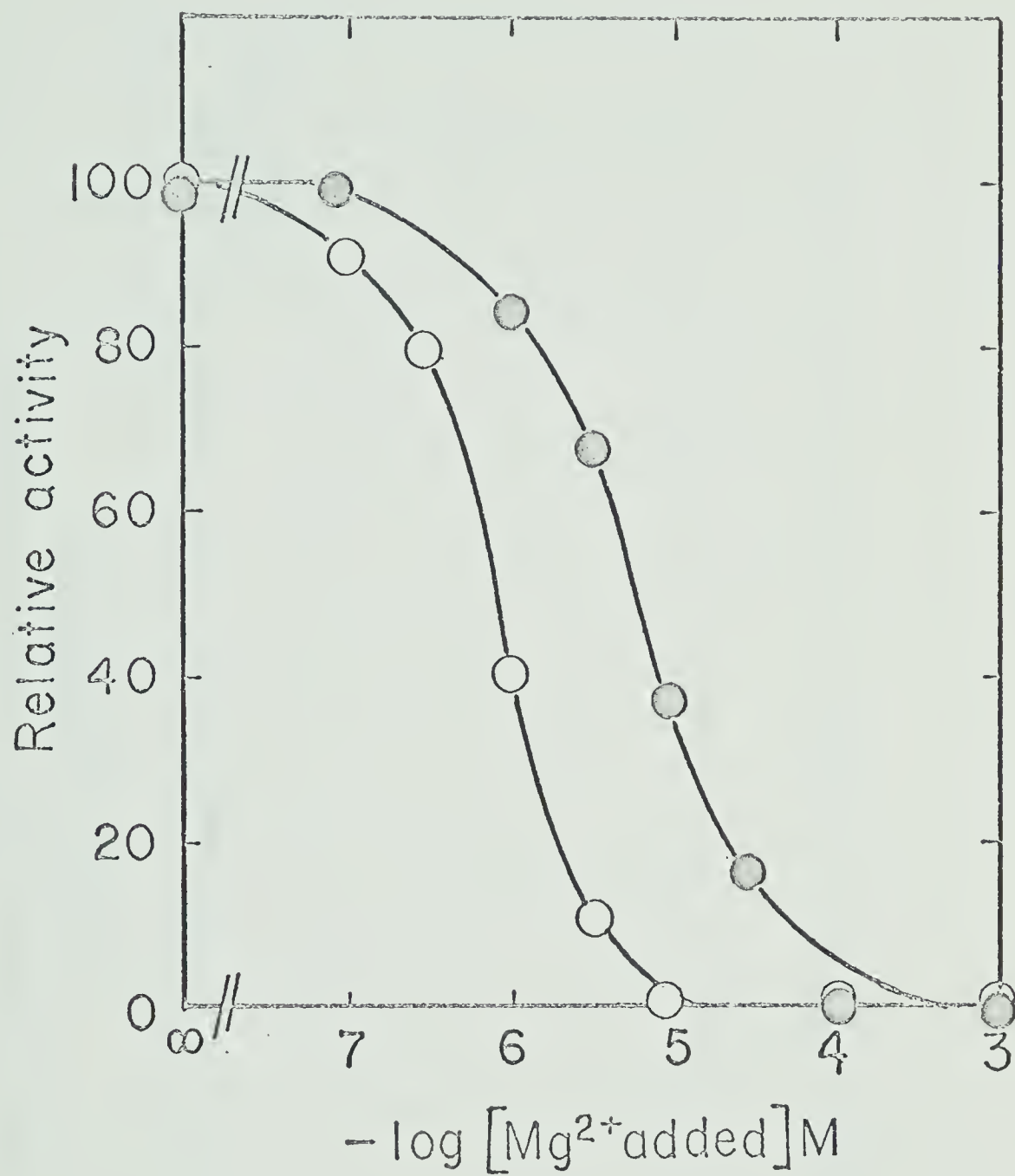


Figure 13. Relative activities for untreated myosin. The symbols are the same as shown in Figure 11. The relative activities were calculated from the data presented in Figure 11.



Table 10

Kapp for Binding of  $Mg^{2+}$  to Myosin Calculated From  
the  $Ca^{2+}$  Binding Reaction

$10^8 \times \{Ca^{2+} \text{ added}\} (M)$	$10^4 \times \{Mg^{2+} \text{ added}\} (M)$	$10^8 \times \{Ca^{2+} \text{ bound}\} (M)$	$10^5 \text{ Kapp}$ for binding of $Mg^{2+}$ ( $M^{-1}$ )
3.0	-	2.4	2.2
3.0	0.1	2.0	3.0
3.0	1.0	1.0	3.0
3.0	3.6	0.5	2.1
3.0	10.0	0.2	0.59

Determinations were carried out in 0.05M KCl, 0.05M tris-Cl (pH 7.4) with 1.0mM ATP at 25°C.



is obtained, presumably because of secondary effects of the bound  $\text{Mg}^{2+}$  on myosin.

As an attempt to detect the  $\text{Mg}^{2+}$  binding to myosin directly, the reaction of calmagite with divalent cations was used to measure the concentrations of  $\text{Mg}^{2+}$  in the filtrate obtained by expressing the buffer solution containing variable amounts of  $\text{Mg}^{2+}$  through a membrane (100 mμ pore size). The addition of myosin to the buffer was shown to reduce the amount of  $\text{Mg}^{2+}$  in the filtrate. Assuming that this reduction is caused by the  $\text{Mg}^{2+}$  binding to myosin, the difference between  $\text{Mg}^{2+}$  contents between the filtrates obtained from the solutions with and without myosin was plotted as a function of the  $\text{Mg}^{2+}$  concentration added to the buffer as shown in Fig. 14. The plot in this figure appears to approximate the curve calculated using a value of  $3.2 \times 10^6 \text{ M}^{-1}$  for the association constant and that of 0.8 for the number of binding sites per mole of myosin (molecular weight of 500,000 daltons) in a binding reaction between  $\text{Mg}^{2+}$  and myosin, which follows the mass action law. The two other curves drawn in Fig. 15 depict the range of affinities which cover the results with a slight alteration in the number of binding sites. There is some difference in magnitude between the binding affinities determined in this way and those calculated from the competition with  $\text{Ca}^{2+}$ , although either set of values show a binding remote from the formation of  $\text{MgATP}^{2-}$ . Fig. 15 shows that the decrease in relative activity follows the amount of  $\text{Mg}^{2+}$  binding, which was calculated by using Kapp values of  $10^6 \text{ M}^{-1}$  for the  $\text{Mg}^{2+}$  binding reaction, and  $1.89 \times 10^4 \text{ M}^{-1}$  for the formation of  $\text{MgATP}^{2-}$ . On the other hand, the  $\text{MgATP}^{2-}$  complex may be as good a substrate as  $\text{ATP}^{4-}$  or  $\text{HATP}^{3-}$



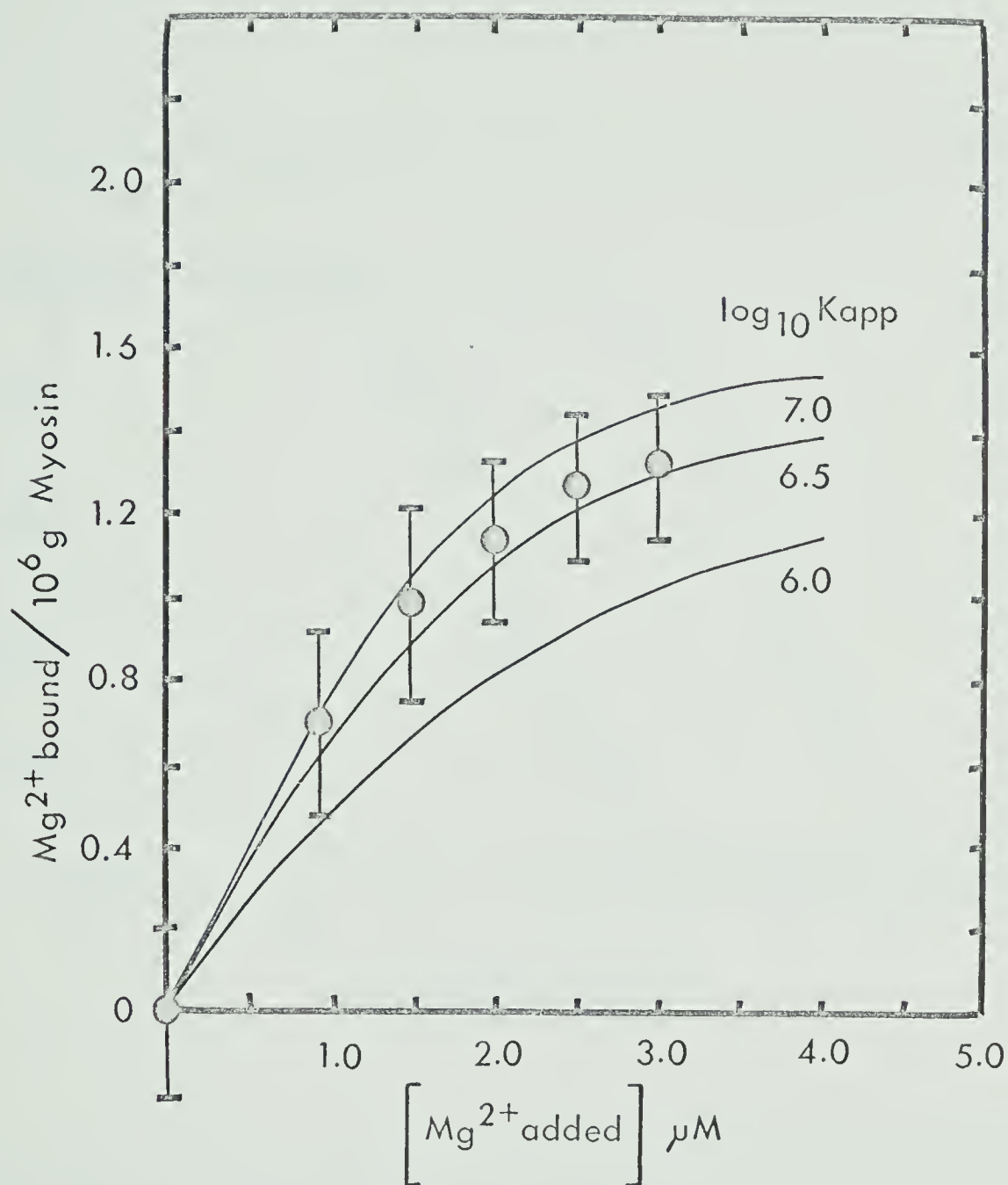


Figure 14: The  $\text{Mg}^{2+}$  binding reaction to myosin using calmagite. Conditions: 0.01M KCl, 0.01M histidine (pH 7.4), 1.0 mg myosin/ml, 25°C. The points indicate the amount of bound  $\text{Mg}^{2+}$  and the curves represent the computed formation of bound  $\text{Mg}^{2+}$  assuming that 0.8 mole  $\text{Mg}^{2+}$  is binding to one mole of myosin (500,000 daltons). The vertical bar in this figure indicates the sum of the deviations due to the range of duplicates in the determination of free  $\text{Mg}^{2+}$  and the standard error of the calibration curve (Fig. 3), which is  $1.0 \pm 0.15 \mu\text{M Mg}^{2+}$ .





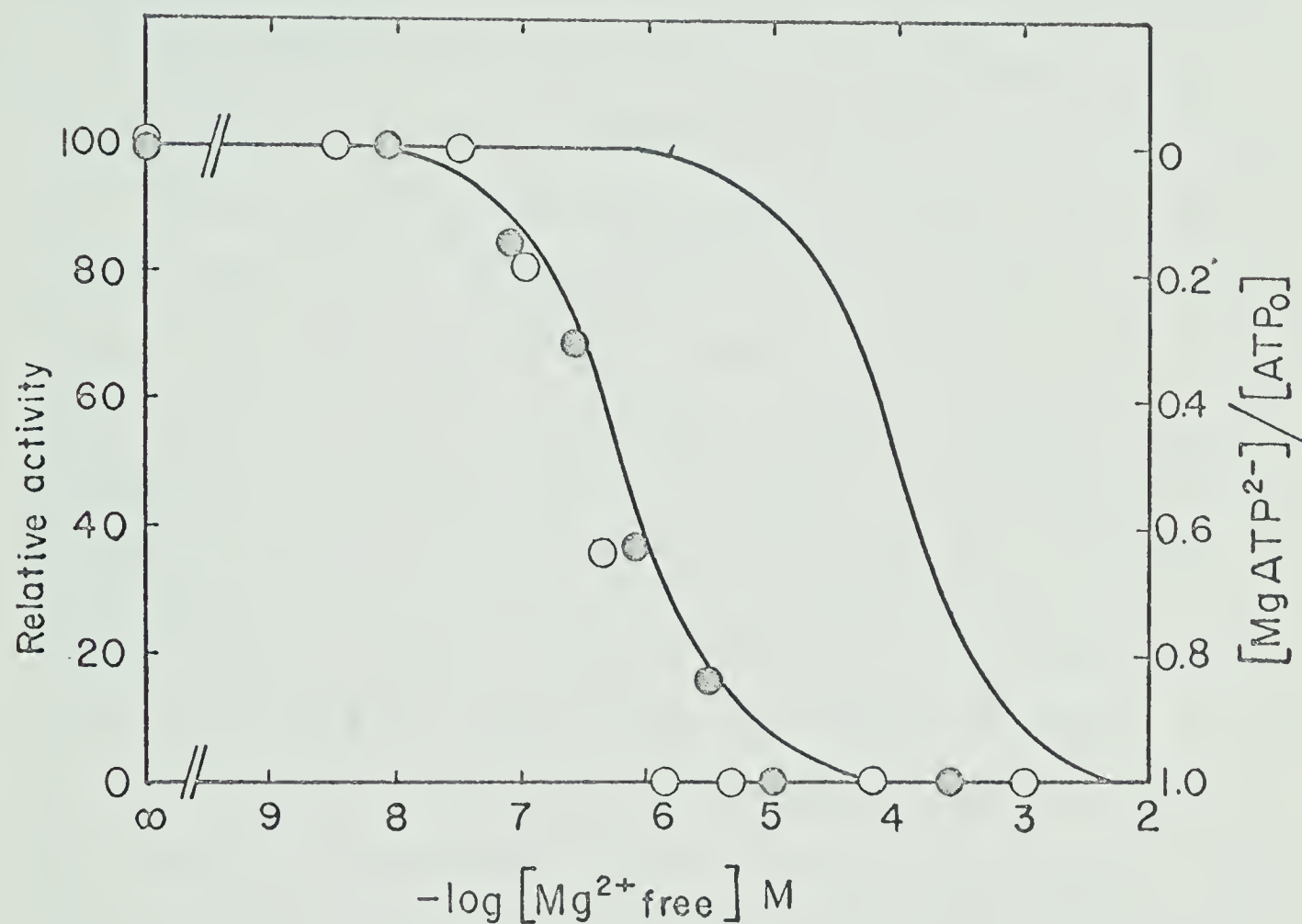


Figure 15. Relative activity of untreated myosin as a function of the calculated concentration of free  $\text{Mg}^{2+}$ . The relative activities were calculated from the data presented in Figure 11. The curve drawn through the experimental points represents binding of  $\text{Mg}^{2+}$  to myosin, and the continuous curve on the right denotes the formation of  $\text{MgATP}^{2-}$ .



since even when essentially all the ATP molecules are in the form of  $\text{MgATP}^{2-}$ , the pMB-modified myosin does not show significant inhibition of the ATPase by  $\text{Mg}^{2+}$  (Fig. 12).

### C. Enzymatic Activity in the Presence of Both Calcium and Magnesium

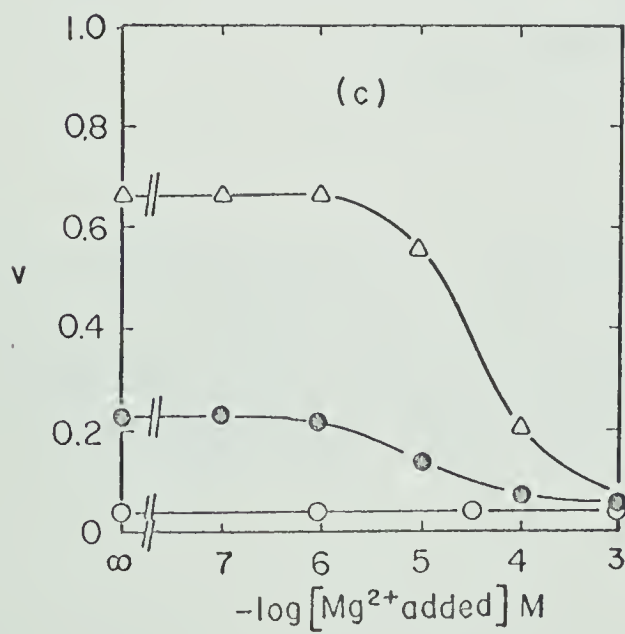
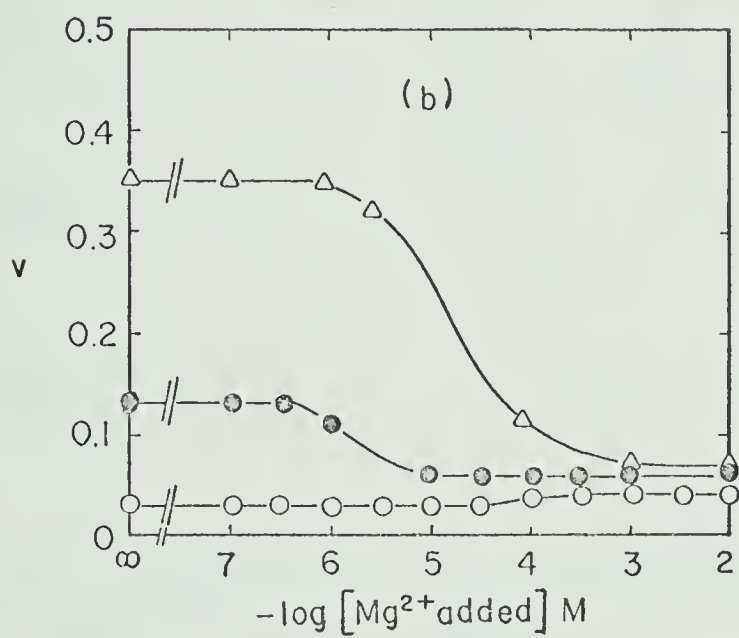
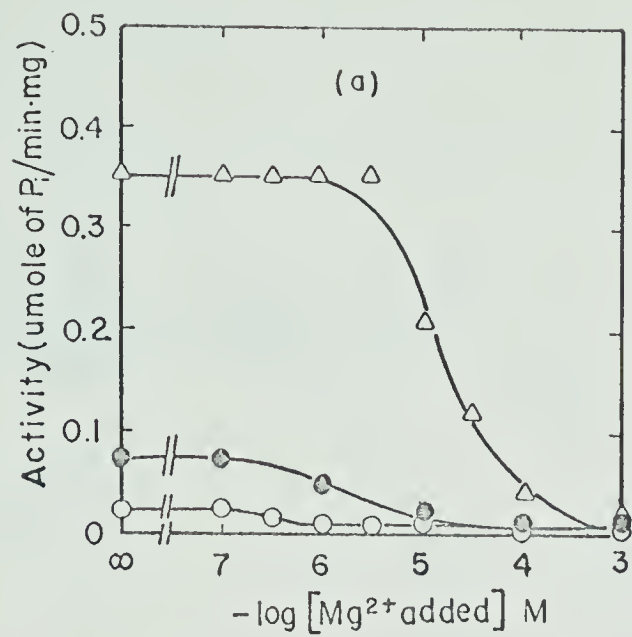
It was suggested above that  $\text{Ca}^{2+}$  affects the NTPase by a weak binding of  $\text{Ca}^{2+}$  to myosin, or the formation of  $\text{CaATP}^{2-}$ , or by both; whereas  $\text{Mg}^{2+}$  inhibits the NTPase by binding tightly to myosin. If so,  $\text{Mg}^{2+}$  should inhibit the  $\text{Ca}^{2+}$  activated NTPase without causing a significant change in the amount of  $\text{Ca}^{2+}$  bound weakly to myosin or to NTP. Fig. 16 shows the effect of  $\text{Mg}^{2+}$  on the ATPase and ITPase activities in the presence of a fixed amount of  $\text{Ca}^{2+}$ . In Fig. 17, the relative activity as defined in Fig. 8 is plotted against the amount of added  $\text{Mg}^{2+}$ . The results in Fig. 17 suggest that the inhibition is not caused by a decrease in the amount of  $\text{CaNTP}^{2-}$  or by the formation of  $\text{MgNTP}^{2-}$ . As the  $\text{Ca}^{2+}$  concentration is increased from 0.032mM to 1.0mM, the amount of  $\text{Mg}^{2+}$  required to inhibit the enzymatic activity also increases. This is interpreted as being due to the competition between  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$  for the binding site in myosin, but not for complex formation with nucleotides, since the calculated changes in amounts of cation-nucleotide complexes do not follow the relationship between enzymatic activity and  $\text{Mg}^{2+}$  concentration. For instance, in the presence of 0.1mM NTP and 1.0mM  $\text{Ca}^{2+}$ , the addition of 0.1mM  $\text{Mg}^{2+}$  causes 90% inhibition of the relative activity, whereas the decrease in  $\text{CaNTP}^{2-}$  concentration is less than 30%, and the formation of  $\text{MgNTP}^{2-}$  amounts to only 7-8% of the total nucleotide present.

Although the ITPase and the pMB-modified ATPase are not inhibited



Figure 16. Enzymatic activities in the presence of  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$ . The NTP concentration was 0.1mM in all cases. (a), ATPase; (b), ITPase; (c), modified myosin ATPase (2.5 moles pMB/ $10^5$  g. myosin): (○), no added  $\text{CaCl}_2$ ; (●),  $3.2 \times 10^{-5}\text{M}$  added  $\text{CaCl}_2$ ; (Δ),  $1.0 \times 10^{-3}\text{M}$  added  $\text{CaCl}_2$ . The protein concentration was 0.33 mg/ml of reaction mixture in the absence of  $\text{CaCl}_2$  and 0.12 mg/ml when  $\text{CaCl}_2$  was added.









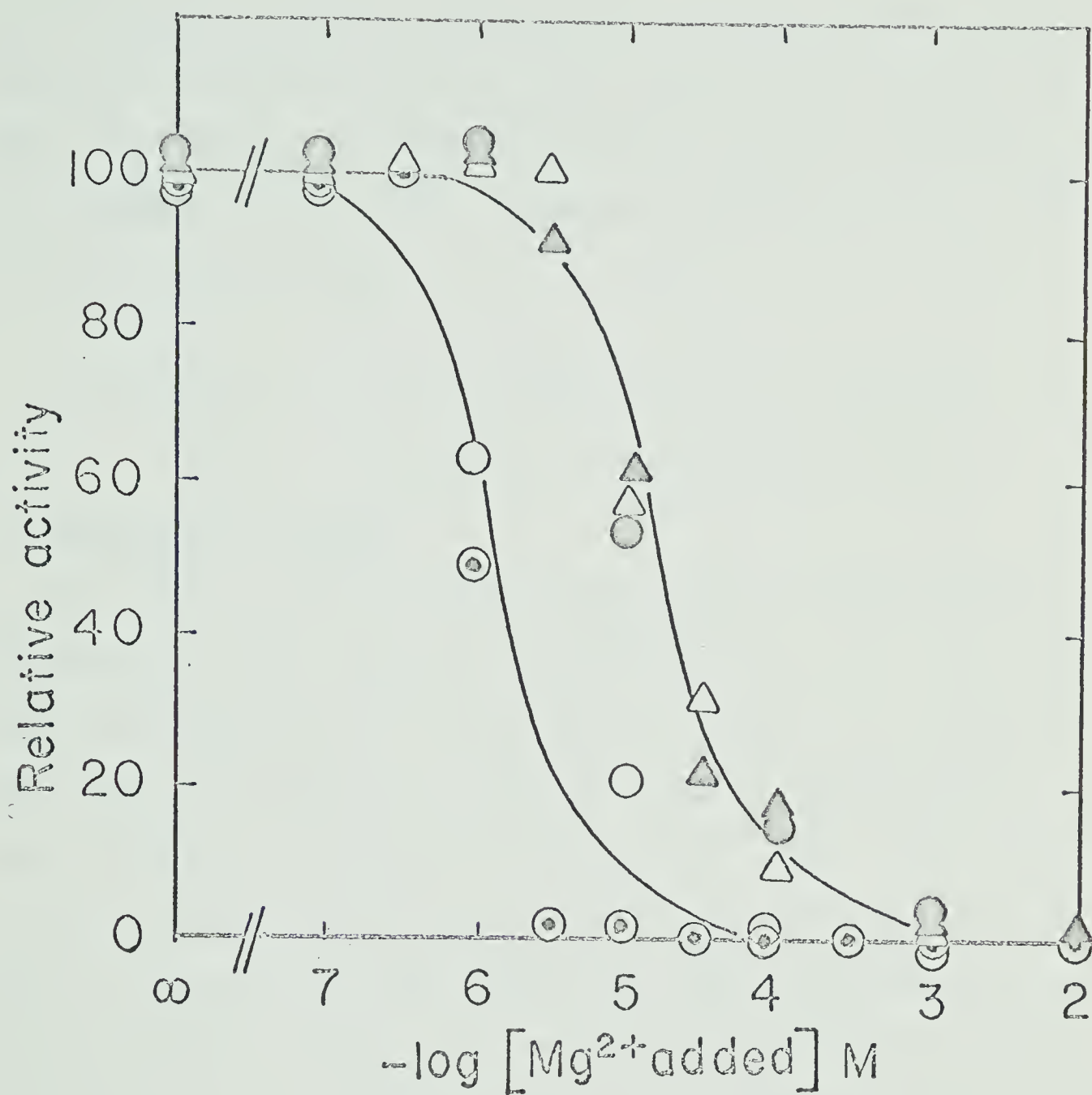
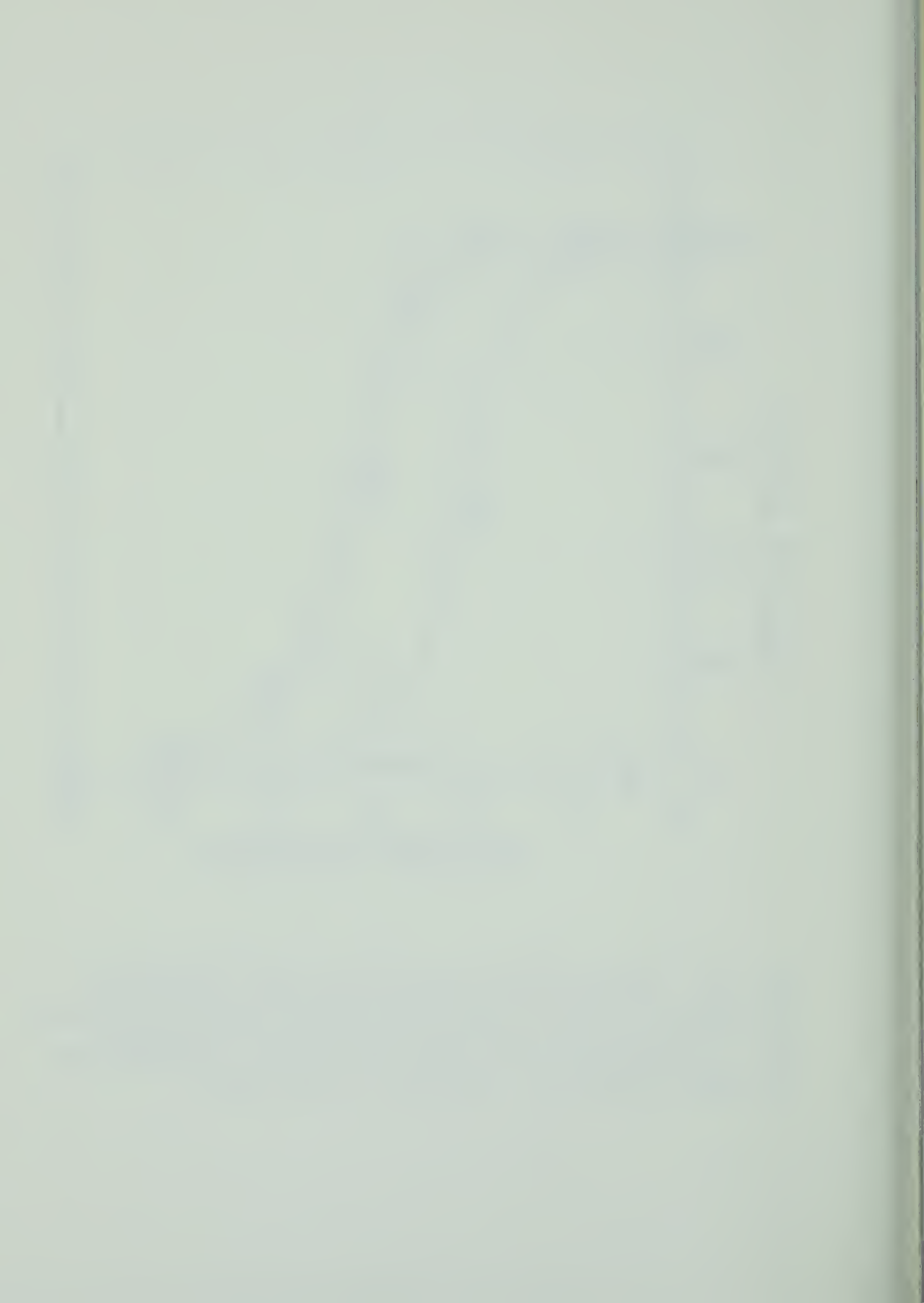


Figure 17. Relative activity (calculated from the results shown in Fig. 15) as a function of the amount of added  $\text{Mg}^{2+}$ . The NTP concentration was  $0.1\text{mM}$  in all cases. The circles indicate the value with  $3.2 \times 10^{-5}\text{M}$  added  $\text{Ca}^{2+}$ , and the triangles with  $1.0 \times 10^{-3}\text{M}$  added  $\text{Ca}^{2+}$ . ( $\circ$ ), ATP; ( $\odot$ ), ITP; ( $\Delta$ ), ATP; ( $\blacktriangle$ ), ITP. Modified myosin ( $2.5$  moles of  $\text{pMB}/10^5 \text{ g.}$ ): ( $\odot$ ), ATP, of myosin ATPase.



by  $\text{Mg}^{2+}$ , they become susceptible to the  $\text{Mg}^{2+}$  effect if activated first by  $\text{Ca}^{2+}$  (Fig. 16b and 16c). The relative activity of ITPase plotted against  $\text{Mg}^{2+}$  coincides with the results obtained with unmodified ATPase (Fig. 17). However, the pMB-modified ATPase shows some resistance to the inhibitory effect of  $\text{Mg}^{2+}$ , suggesting a weakened binding of  $\text{Mg}^{2+}$  to the pMB-modified myosin.

## 2. The Effects of Calcium and Magnesium on the Actomyosin

### Adenosine Triphosphatase and Superprecipitation

#### A. Activation by Calcium

The effect of various concentrations of  $\text{Ca}^{2+}$  on the actomyosin ATPase activity was analysed at low concentrations of KCl, using the same procedure as for the analysis of myosin ATPase. The chelating agent, EDTA, was added to the reaction mixture to eliminate the effects of divalent cations when necessary (Nihei, 1967). The time course of ATP hydrolysis in the presence of  $\text{Ca}^{2+}$  shows a curvature which appears to consist of two phases (Fig. 18). This was not observed with the myosin ATPase. Plotting the reaction rate from both the initial and slow phases of the activity shows a  $\text{Ca}^{2+}$  activation of the ATPase reaching a maximum at 10mM added  $\text{Ca}^{2+}$  (Fig. 19), in fair agreement with the  $\text{Ca}^{2+}$ -ATPase of myosin.

In conjunction with the determinations of ATPase, the rate and extent of superprecipitation were evaluated. From the change in turbidity recorded with time, the rate of superprecipitation was taken as the reciprocal of the time required to reach 50% of the maximum extent ( $t_{1/2}$ ). The extent here is expressed by the difference between the



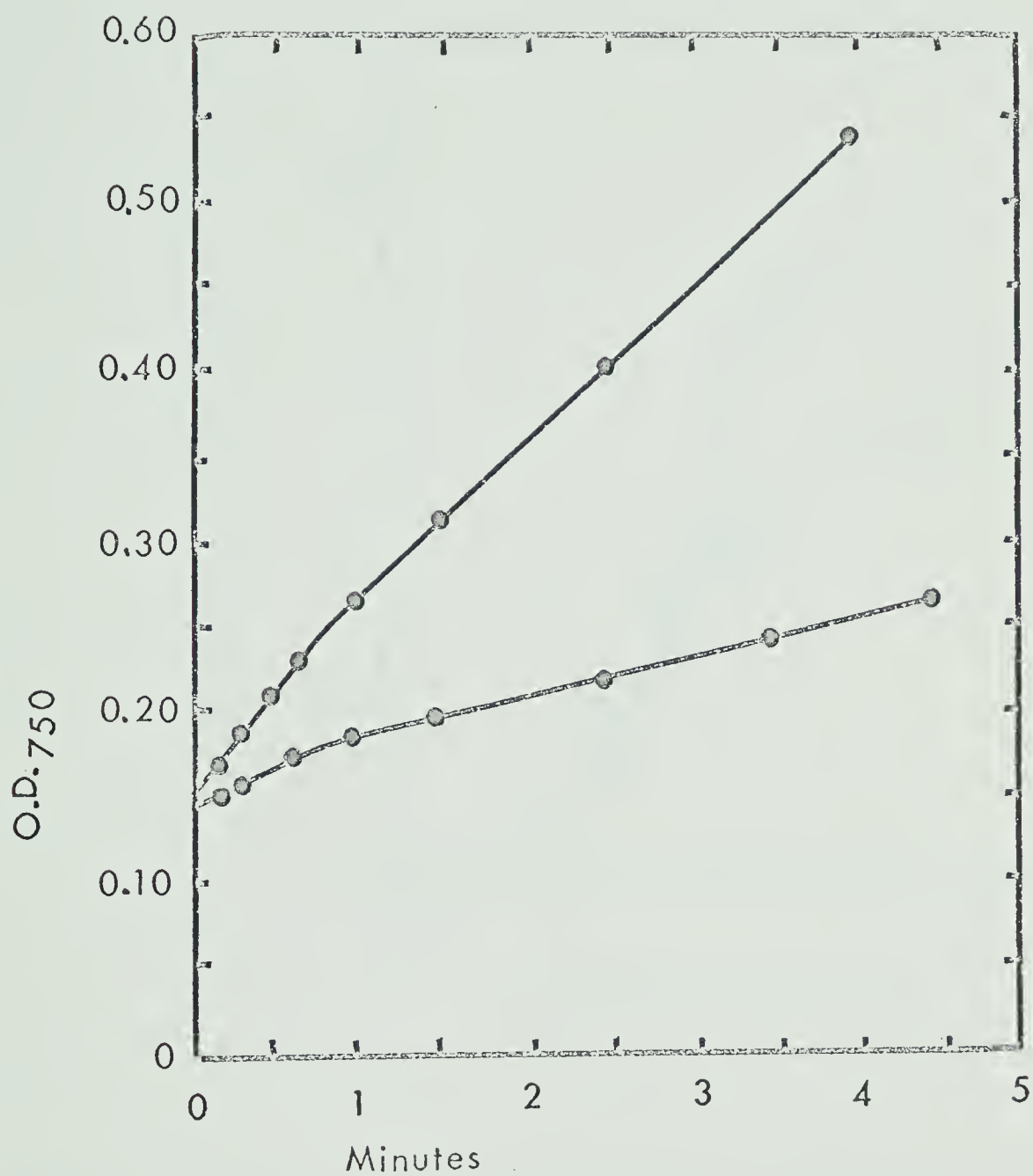
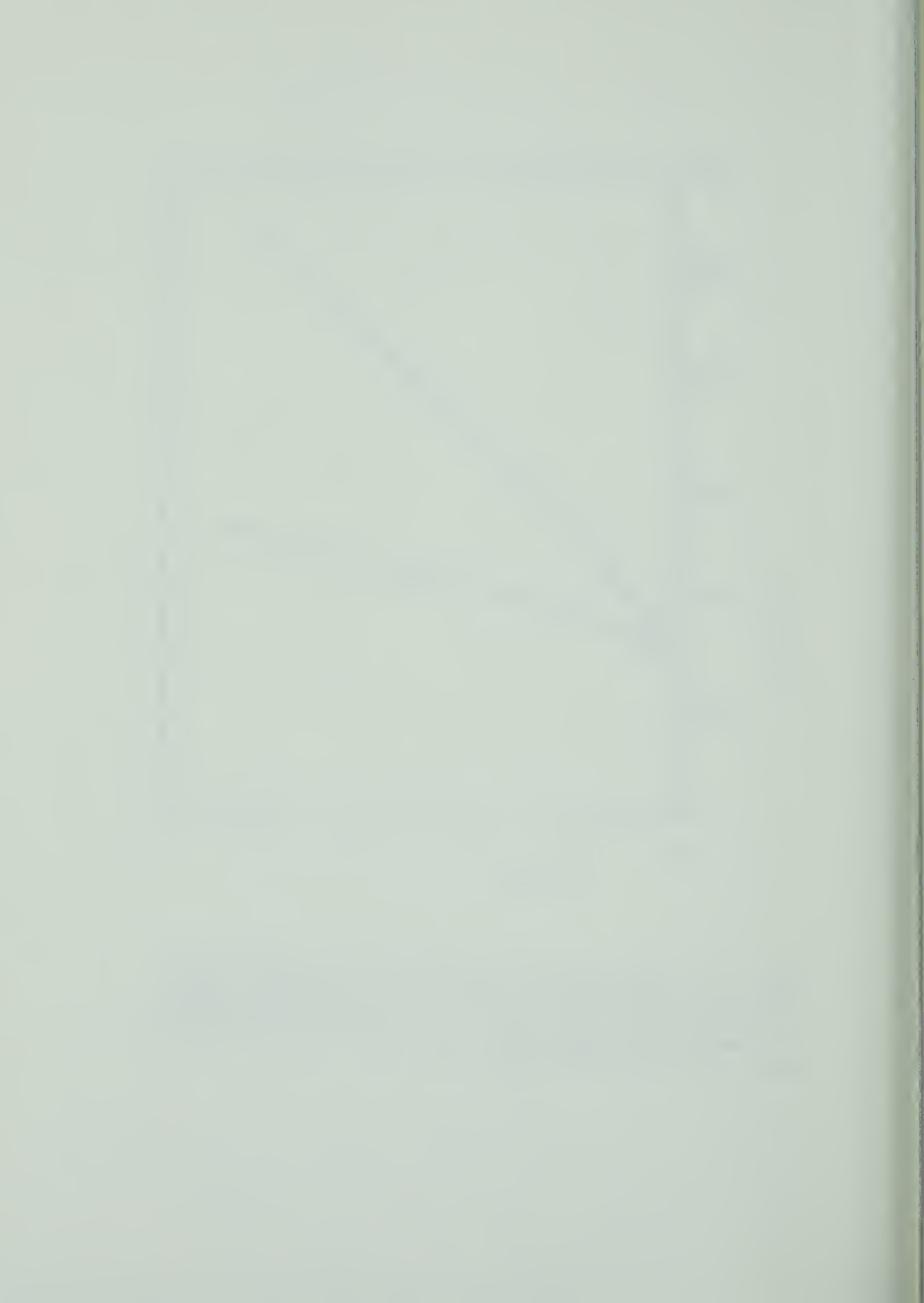


Figure 18. Time course of the  $\text{Ca}^{2+}$ -ATPase of actomyosin at different concentrations of added  $\text{CaCl}_2$ . Condition: 0.005 - 0.012M KCl, 0.05M tris-Cl (pH 7.4), 1.0mM ATP, 1.0mM EDTA, 0.188 mg actomyosin/ml, 25°C. Lower curve,  $1.5 \times 10^{-3}\text{M}$   $\text{CaCl}_2$ , upper curve,  $1.0 \times 10^{-2}\text{M}$   $\text{CaCl}_2$ .



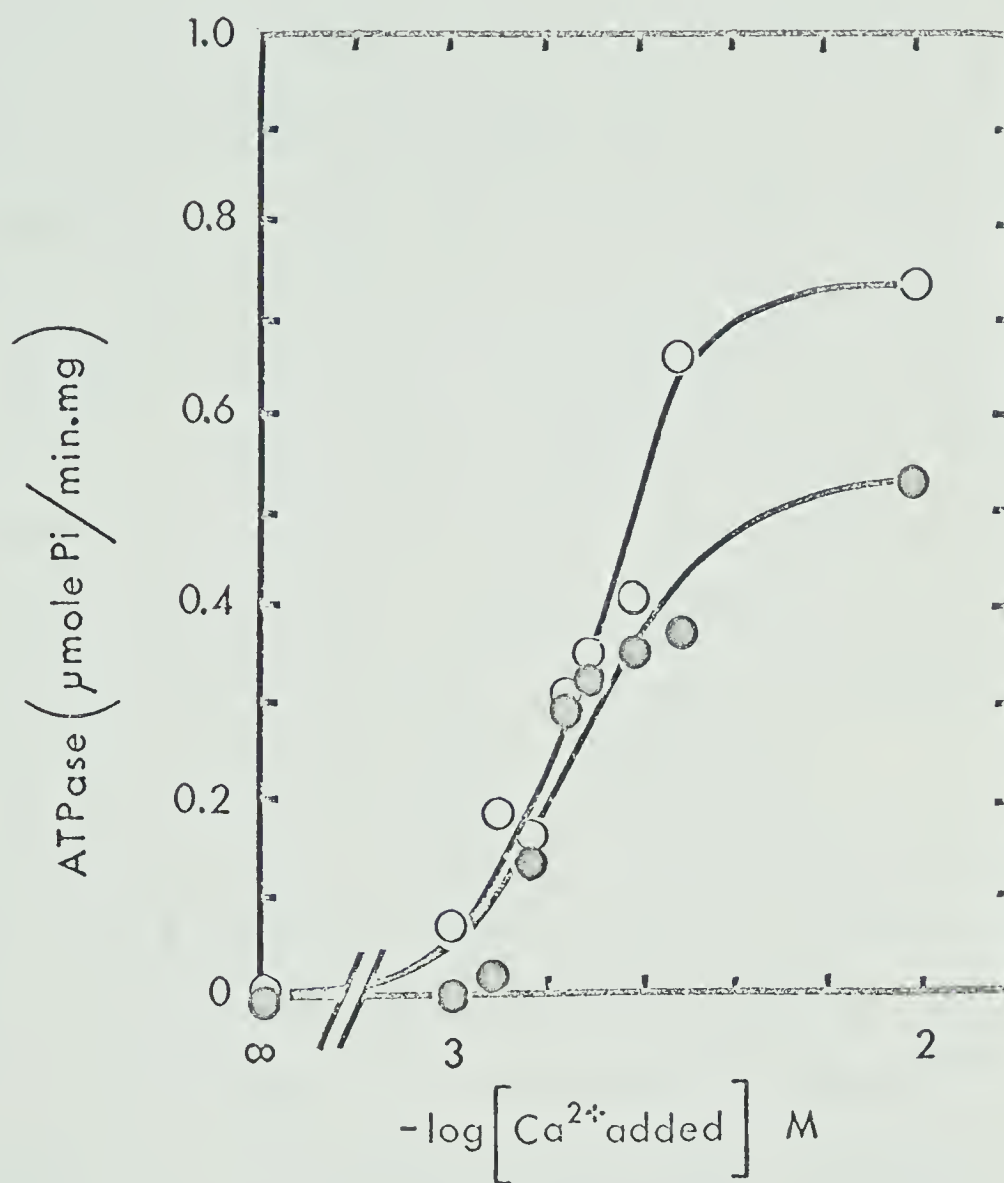
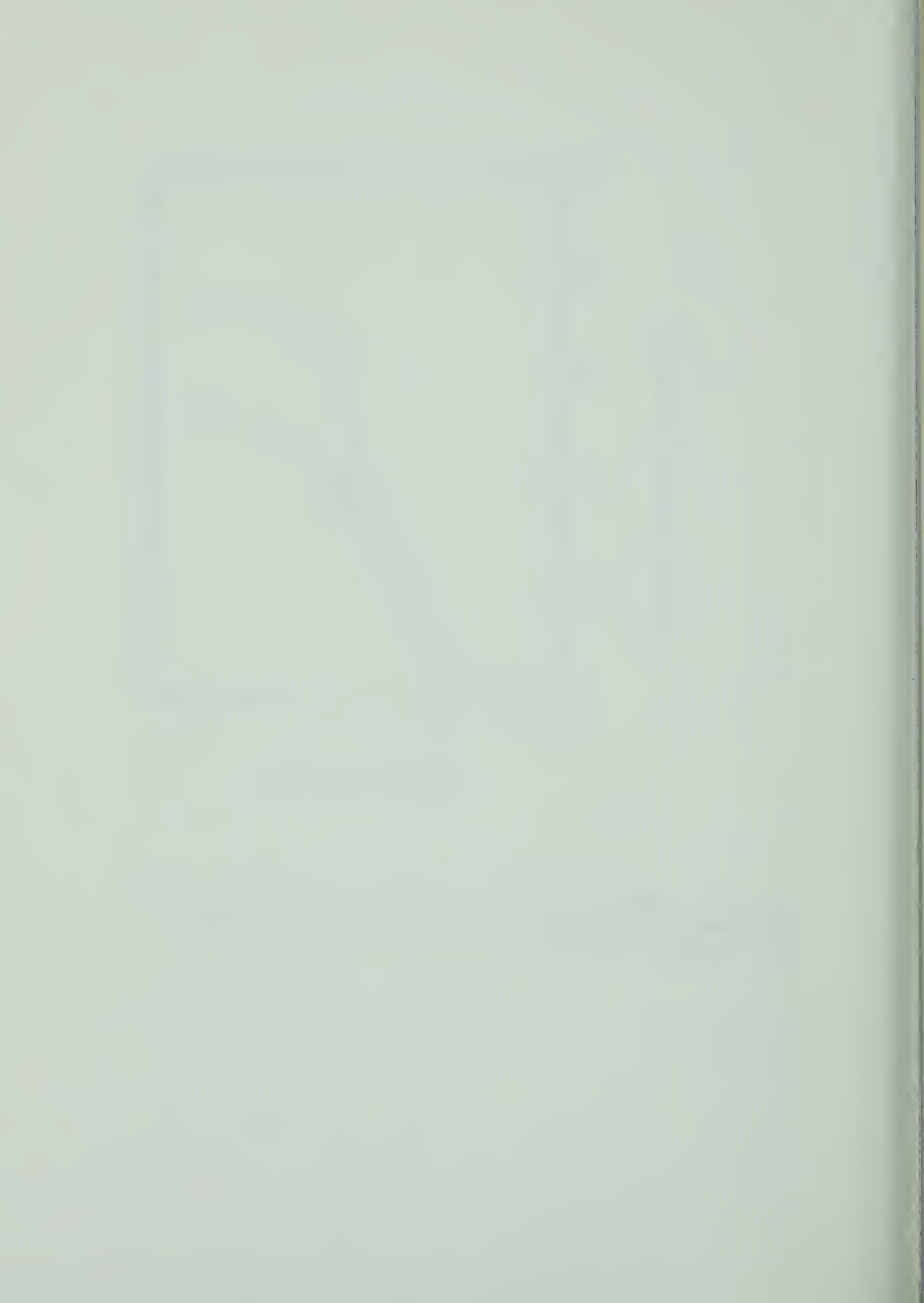


Figure 19: Effects of  $\text{Ca}^{2+}$  on the ATPase of actomyosin. Same conditions as in Figure 17. (○), ATPase, initial phase; (●), ATPase, late phase.





absorbances of the fully superprecipitated actomyosin and the starting solution. The time course of  $\text{Ca}^{2+}$  activated superprecipitation resembles the previous results for superprecipitation (Nihei and Yamamoto, 1969), but from the values of the time constant ( $t_{1/2}$ ), the  $\text{Ca}^{2+}$  activation is clearly more sluggish than the  $\text{Mg}^{2+}$  effect (Table 11), where the  $t_{1/2}$  value is about one second for optimal concentrations of  $\text{Mg}^{2+}$ .

From a comparison of the rate of superprecipitation with the ATPase activity, it appears that the aggregate formation of actomyosin retards the diffusion of substrate and/or product, which becomes the rate limiting step. The initial activity is not the pre-steady state burst (Kinoshita et al., 1969; Lymn and Taylor, 1970) which occurs at a time in the order of milliseconds.

The experimental data for ATPase and superprecipitation are expressed as relative activity, using the formula previously found successful for the analysis of the myosin NTPase activity. The relative activity was plotted against the concentration of free  $\text{Ca}^{2+}$  to test the possibility of a  $\text{Ca}^{2+}$  binding reaction being responsible for the activation. The free  $\text{Ca}^{2+}$  was calculated as that which is not bound to ATP or EDTA, using the known association constants for ATP (Table 7) and EDTA (Chaberek and Martell, 1959). From a comparison of the plot in Fig. 20 to the myosin ATPase data (Fig. 8), it seems that actin has not altered the nature of the  $\text{Ca}^{2+}$  effect on myosin. The activation of actomyosin ATPase may thus be interpreted as being a weak  $\text{Ca}^{2+}$  binding reaction, or a more rapid hydrolysis of the  $\text{CaATP}^{2-}$  complex. The extent of superprecipitation is about the same at various concentrations of  $\text{Ca}^{2+}$ , with a slight increase at 10mM  $\text{CaCl}_2$ . This increase



Table 11

The Rate of Superprecipitation of Actomyosin  
in the Presence of  $\text{Ca}^{2+}$

<u><math>\{\text{Ca}^{2+} \text{ added}\}</math></u> <u>mM</u>	<u><math>t_{1/2}</math></u> <u>seconds</u>
1.00	1,070*
1.25	54.3
1.50	18.3
1.75	16.5
2.00	17.4
1.50	12.7
3.00	8.6
9.00	8.0
10.00	8.0

Determinations were carried out in 0.005 - 0.012M KCl, 0.05M tris-Cl (pH 7.4), 1.0mM ATP, 1.0mM EDTA, 0.02 mg actomyosin/ml.

\*The maximum extent was not recorded, but assumed to be the same as in the presence of 1.25mM  $\text{CaCl}_2$ .



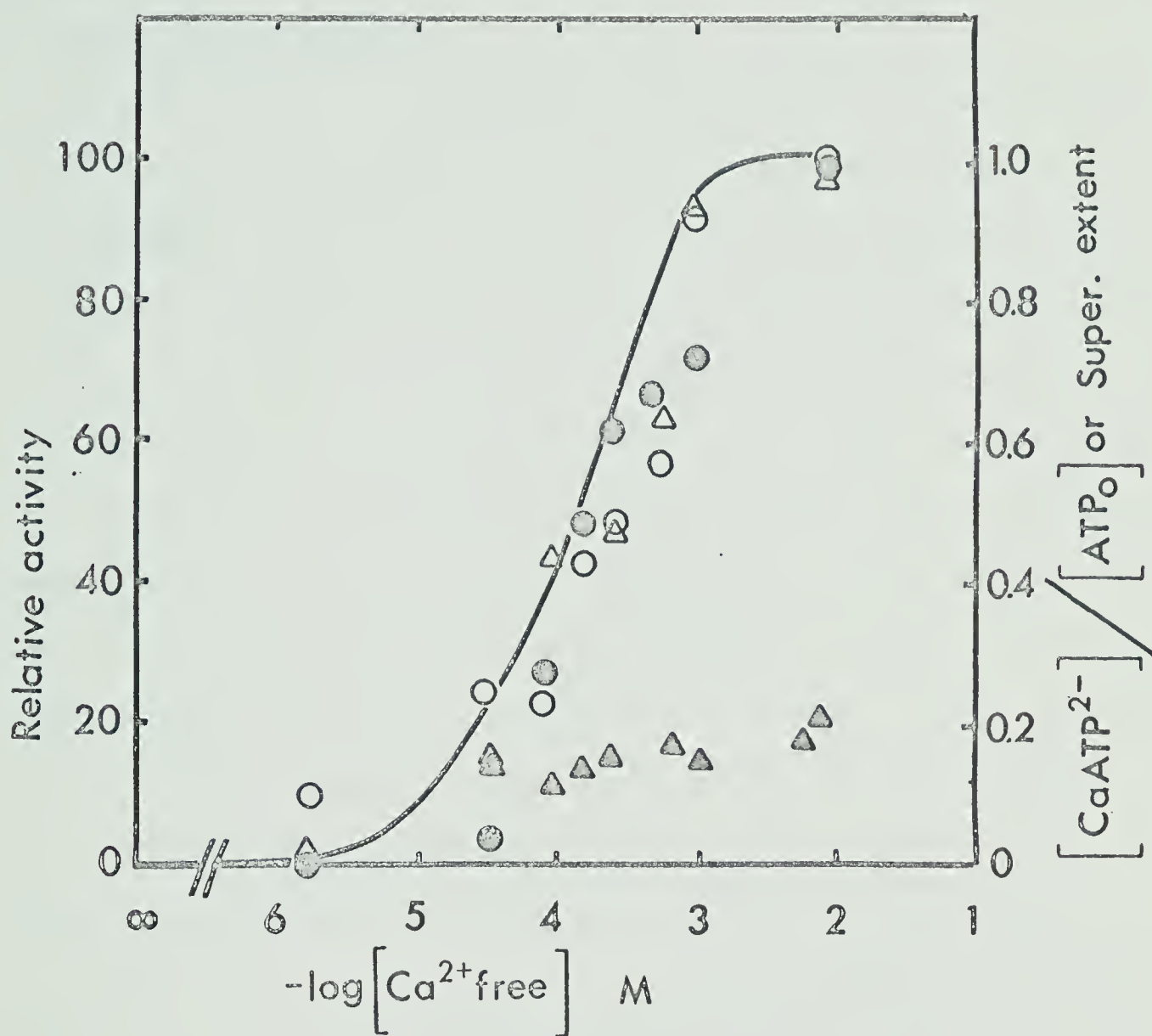


Figure 20: Relative activity of the effects of  $\text{Ca}^{2+}$  on the ATPase and superprecipitation of actomyosin. (○), ATPase, initial phase; (●), ATPase, late phase; (△), rate of superprecipitation; (▲), superprecipitation extent.



is probably due to a secondary precipitation of myosin by  $\text{CaCl}_2$  which has been found to occur at this concentration (Szent-Györgyi, 1951).

#### B. Activation by Magnesium

The  $\text{Mg}^{2+}$  activation of actomyosin ATPase occurs at concentrations of  $\text{Mg}^{2+}$  lower than was required for the  $\text{Ca}^{2+}$  activation (Fig. 21). It is important to measure the activity at low KCl concentrations, since at 0.1M KCl, added  $\text{Mg}^{2+}$  in the concentration range of 0.1-1.0mM inhibits the ATPase. In addition to natural actomyosin, a mixture consisting of a 10 molar excess of actin over myosin was used. Under the conditions of the experiment, over 80% of the myosin is bound to actin, as will be shown in the section on actin-myosin combination. Using the same mixture of actin and myosin, the ATPase activity was determined at various concentrations of ATP with an excess of  $\text{Mg}^{2+}$  (5.0mM). From a reciprocal plot of the experimental data, it is observed that the activity follows Michaelis-Menten kinetics (Fig. 22) with a value for  $K_m$  of 0.05mM, compatible with that previously estimated by Schiselfeld et al., (1970). The relative activity of actomyosin ATPase is calculated from the results of Fig. 21, as previously described. The plot of Fig. 23 suggests that the binding of  $\text{Mg}^{2+}$  to actomyosin is responsible for the activation as in the case of the inhibition of myosin. These findings are also in fair agreement with those of Nihei (1967). In consequence, an attempt was made to determine the  $\text{Mg}^{2+}$ -binding to actomyosin. This was unsuccessful however, in that too much deviation occurred in the experimental data to make any conclusions about the magnitude of the binding constant or the number of sites. According to the result shown in Fig. 24, there is no detectable binding of  $\text{Mg}^{2+}$  to





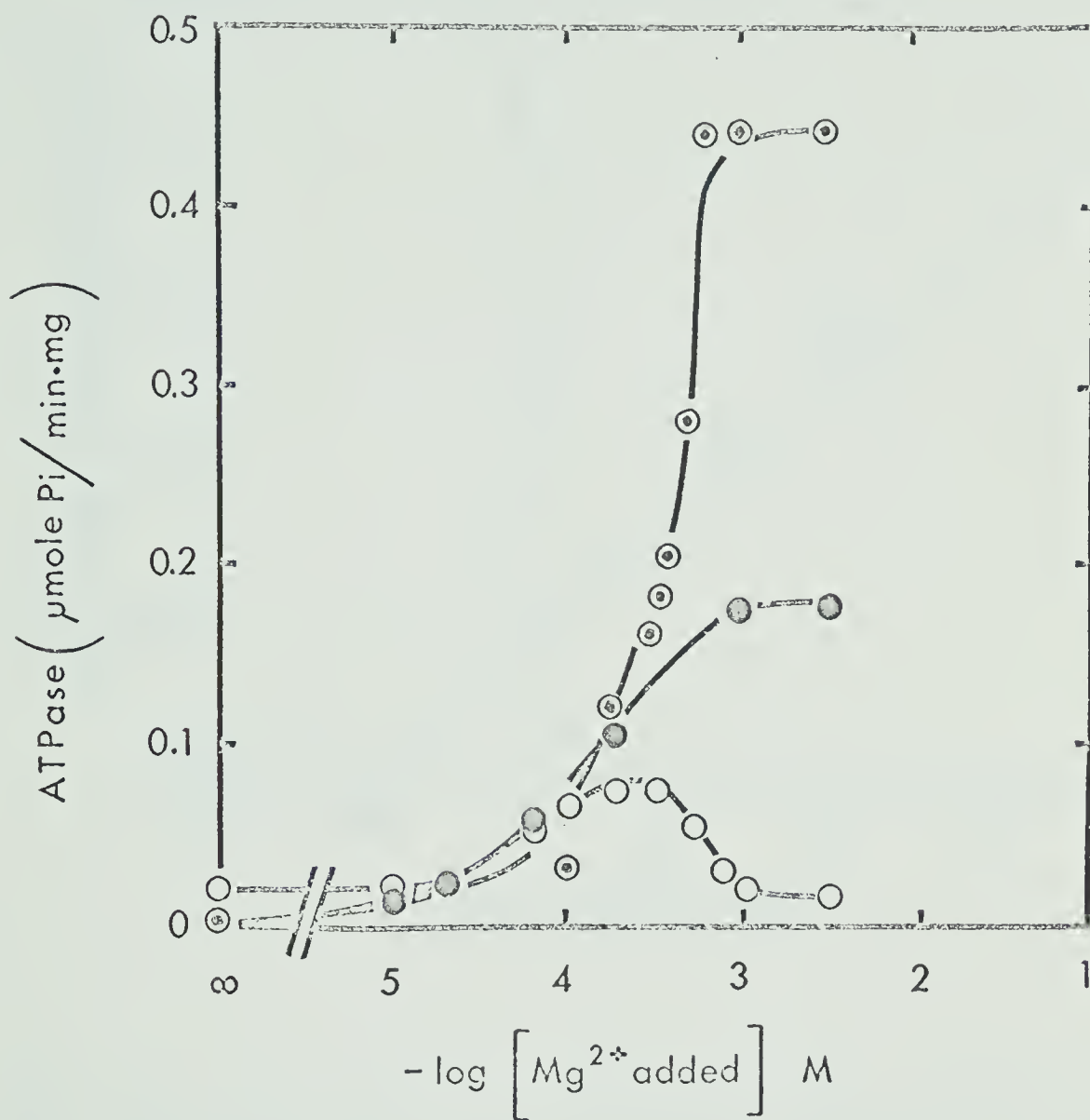


Figure 21: The effect of  $Mg^{2+}$  on actomyosin at different KCl concentrations. ATPase: 0.02M tris-Cl (pH 7.4), 1.0mM EDTA, 25°C. Natural actomyosin, 1.0mM ATP, 0.19 mg/ml: ( O ), 0.1M KCl; ( ◐ ), 0.012M KCl. Reconstituted actomyosin, 0.1mM ATP, 0.11 mg actin/ml, 0.09 mg myosin/ml (10:1 actin to myosin molar ratio, activity calculated per mg. myosin): ( ⊙ ), 0.014M KCl.



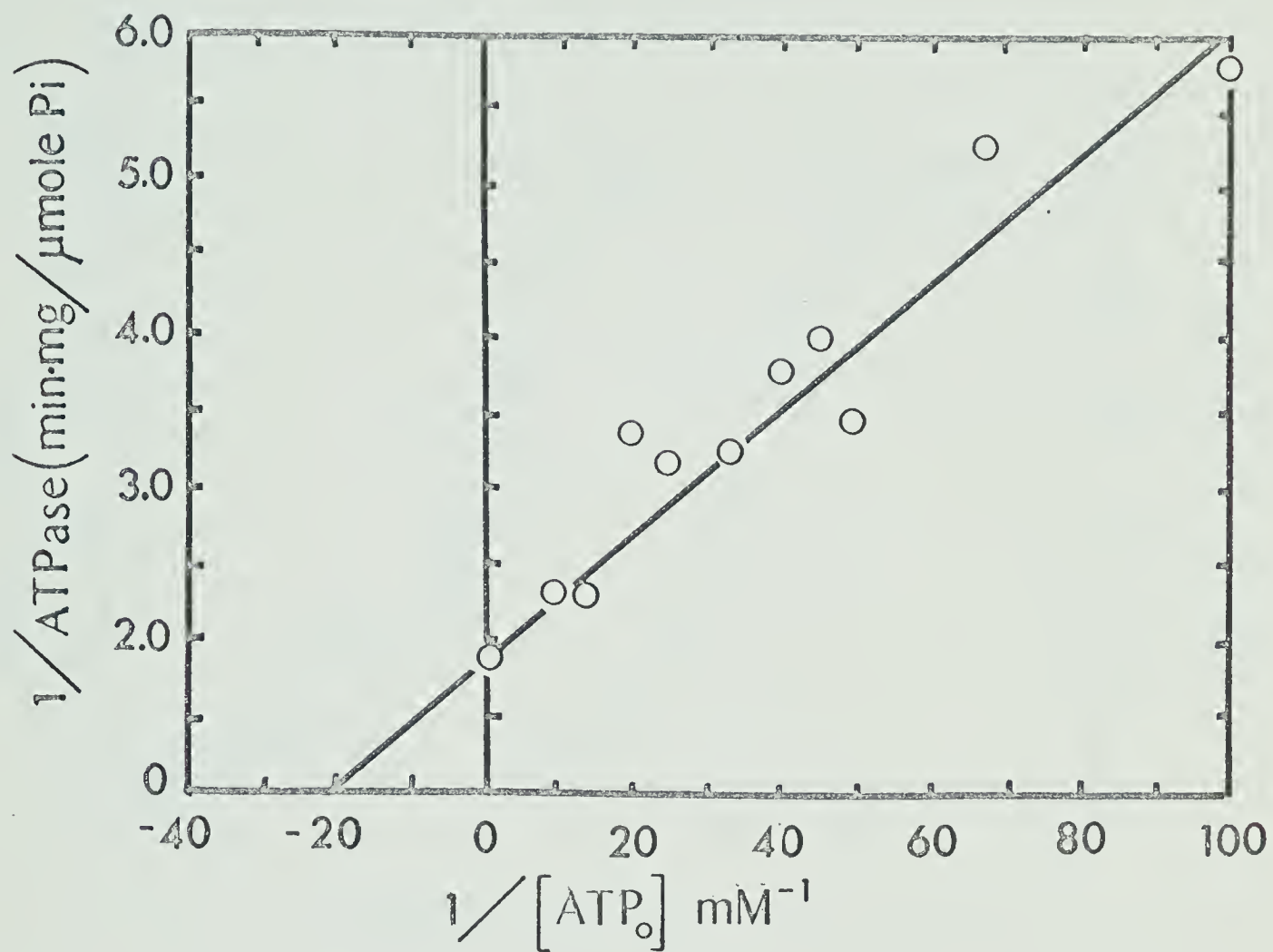


Figure 22: Reciprocal plot of actomyosin ATPase. Conditions: 0.014M KCl, 0.02M tris-Cl (pH 7.4), 5.0mM  $\text{MgCl}_2$ , 1.0mM EDTA, 0.11 mg/ml, 0.09 mg myosin/ ml, 25°C.



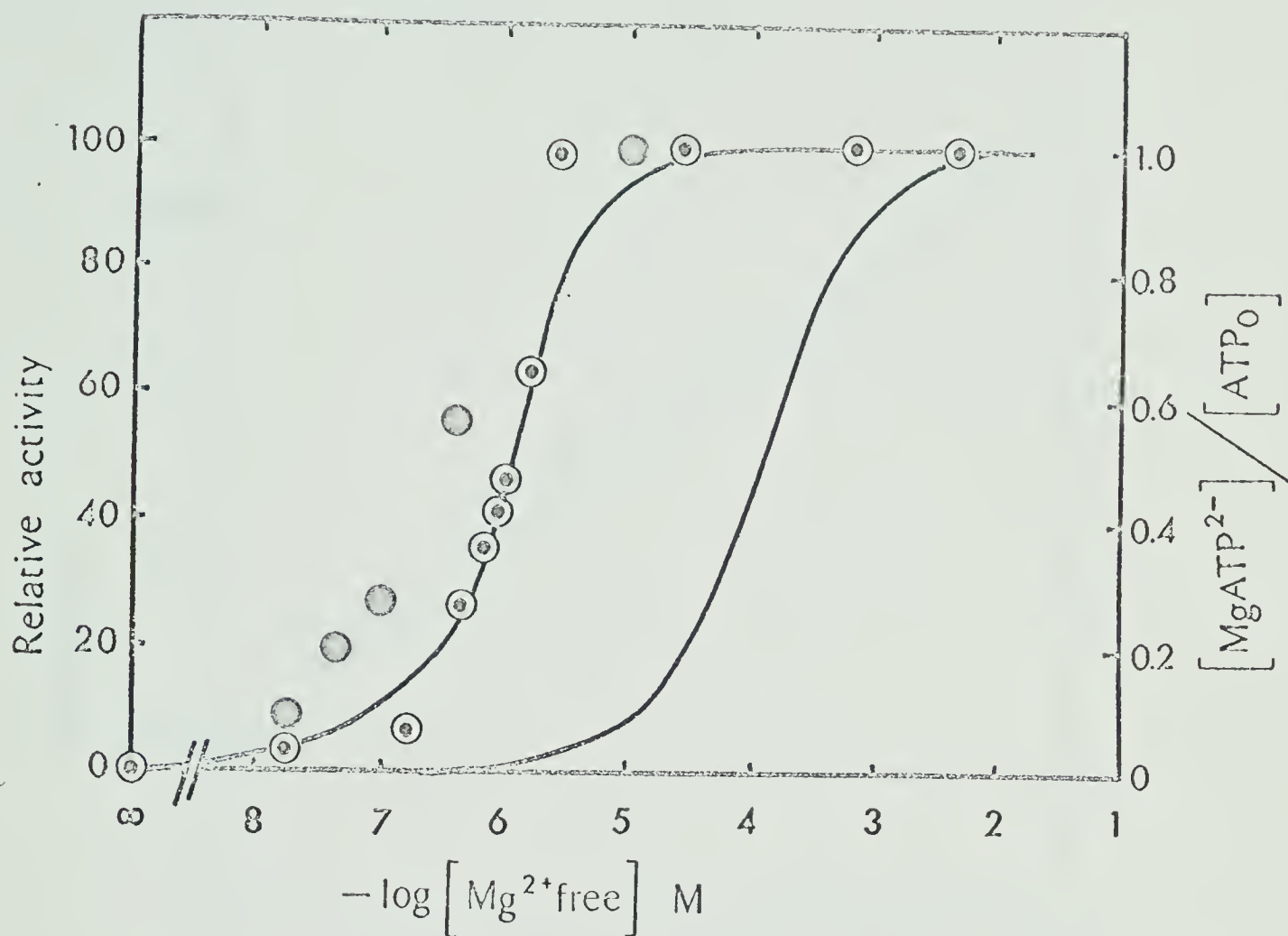


Figure 23: Relative activity of actomyosin as a function of the calculated free  $\text{Mg}^{2+}$ . The symbols are the same as in Figure 20. The curve drawn through the experimental points represents the binding of  $\text{Mg}^{2+}$  to actomyosin and the continuous curve to the right depicts formation of  $\text{MgATP}^{2-}$ .



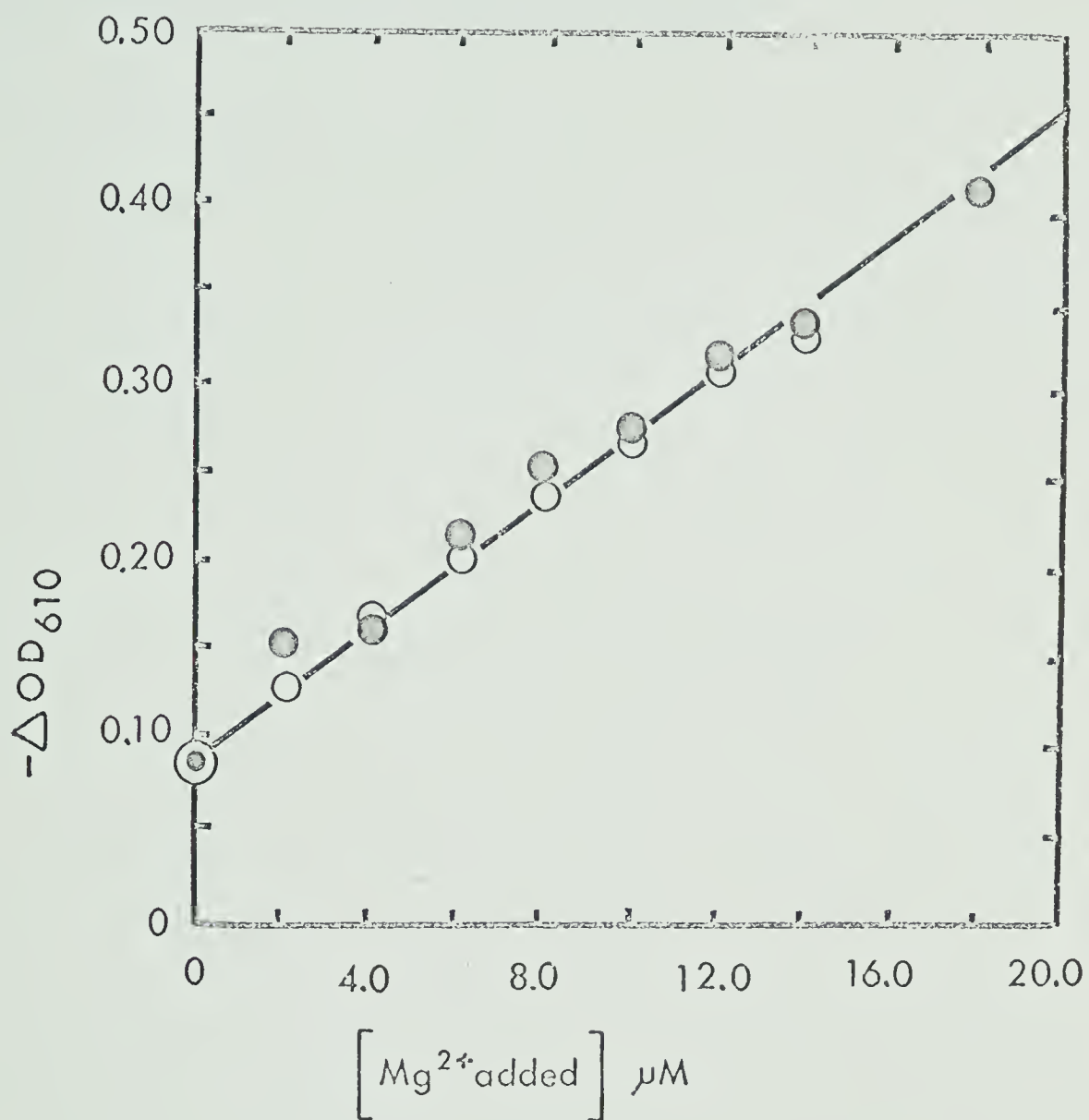


Figure 24: The  $\text{Mg}^{2+}$  binding reaction to F-actin using calmagite. Conditions: 0.1M KCl, 0.01M histidine (pH 7.4), 0.28 mg F-actin/ml, 25°C. (○), total  $\text{Mg}^{2+}$ ; (●), free  $\text{Mg}^{2+}$  in filtrate.





F-actin within the range of  $\text{Mg}^{2+}$  concentrations studied. Therefore any detectable binding which occurs in a mixture of actin and myosin can be regarded as that occurring to myosin.



### 3. The Preparation of the Papain-Hydrolyzed Subfragment of Myosin

Mueller and Perry (1961, 1962) demonstrated for the first time, that the heavy meromyosin fragment prepared by tryptic digestion of myosin, can be further hydrolyzed by trypsin to yield a subfragment of smaller molecular weight which is capable of binding with actin and of hydrolyzing ATP. The observations of the tryptic subfragment with hydrodynamic techniques and electron microscopy indicated that it is a globular particle with a molecular weight ranging from 100,000-170,000 (Mueller and Rice, 1964; Young et al., 1965; Mueller, 1965). As mentioned earlier, it has been reported that the subfragment comes from the thickened end of the myosin molecule. In recent years, the papain digestion of myosin has been shown to release the subfragment from myosin in a single digestion step (Kominz et al., 1965). By using an insoluble cellulose-papain complex, the digestion is easily terminated by centrifugation or ultrafiltration. The light meromyosin portion of the digest can be separated by centrifugation at low ionic strength and the subfragment may be subsequently isolated from the supernatant by Sephadex chromatography (Nihei and Kay, 1968; Lowey et al., 1969), or by the centrifugation of an actin-subfragment complex (Mueller and Perry, 1962; Tada et al., 1969).

The size of the subfragment from the papain digestion of myosin has been estimated as ranging from 110,000-140,000 daltons (Kominz et al., 1965; Nihei and Kay, 1968; Lowey et al., 1969).

As suggested by Lowey et al., (1969), the population of active



subfragment (HMM-S1) molecules is likely to be heterogeneous due to an under- or over-digestion of the tail region of myosin or an internal proteolysis of active subfragment. Also, the active subfragment is well known for its tendency to aggregate.

Jones and Perry (1966) reported that prolonged digestion with trypsin attacks the actin-combining site without affecting the ATPase activity, leading to the conclusion that these two properties of myosin are due to different sites on the subfragment molecule.

The experiments described in this section were carried out to obtain a homogeneous preparation of the myosin subfragment which could be used for the detailed analysis of interactions between actin and the subfragment under various experimental conditions.

In the early stage of the experiments, the subfragment was prepared from digested myosin which had released four equivalents of  $H^+$  per  $10^5$  g of protein mass during 10 minutes of reaction. This process was shown to convert one-third of the myosin mass into a chromatographically separable single component from which the ATPase activity (Nihei and Kay, 1968) of the parent molecule can be recovered. Thereafter, the hydrolysis slowed down, presumably because less-susceptible regions remained for proteolytic attack (Mihalyi and Harrington, 1959). In some experiments, preparations were made using a longer digestion period.

After the removal of insoluble light meromyosin by centrifugation, the digest was concentrated and chromatographed in a descending fashion on a Sephadex G-75 column in order to see if a significant proportion of the digested mass could permeate into



this gel. However, this seemed unlikely, since Fig. 25 shows that the majority of the protein material emerges in the void volume with a small amount of heterogeneous material appearing in later fractions. On the other hand, the chromatographic analysis on a column of Sephadex G-200 superfine exhibited a slight resolution of the main peak (Fig. 26). The protein concentration for these fractions was determined at 280 m $\mu$ , where the subfragment extinction coefficient was taken as 770 cm<sup>2</sup>/g (Young et al., 1965). The right hand peak of the chromatogram (hatched area), representing lighter material, was found to contain most of the ATPase active component.

Rechromatography of the pooled fractions (hatched area) resulted in the pattern depicted in Fig. 27, showing most of the protein in a single peak, but also showing a certain amount of heavier material. From previous studies on papain-digested myosin, a single peak isolated by chromatography in this way was found to show two peaks in the ultracentrifuge (Kominz et al., 1965). One of these two components was subsequently shown to combine with actin, hydrolyze ATP and sediment with  $S_{20,w}^0$  of 2.7 (Lowey et al., 1969). The latter is believed to represent the tail portion of heavy meromyosin (Lowey et al., 1969) and has a high helical content. However, it is water soluble like subfragment and could only be removed from the mixture by chromatographic recycling which was found to be quite tedious. On the other hand, if F-actin was added to a myosin digest, the resulting actin-subfragment could be separated by centrifugation. Thus the subfragment separation should be accomplished in a single step (Mueller and Perry, 1962). In conjunction with this procedure, it was attempted





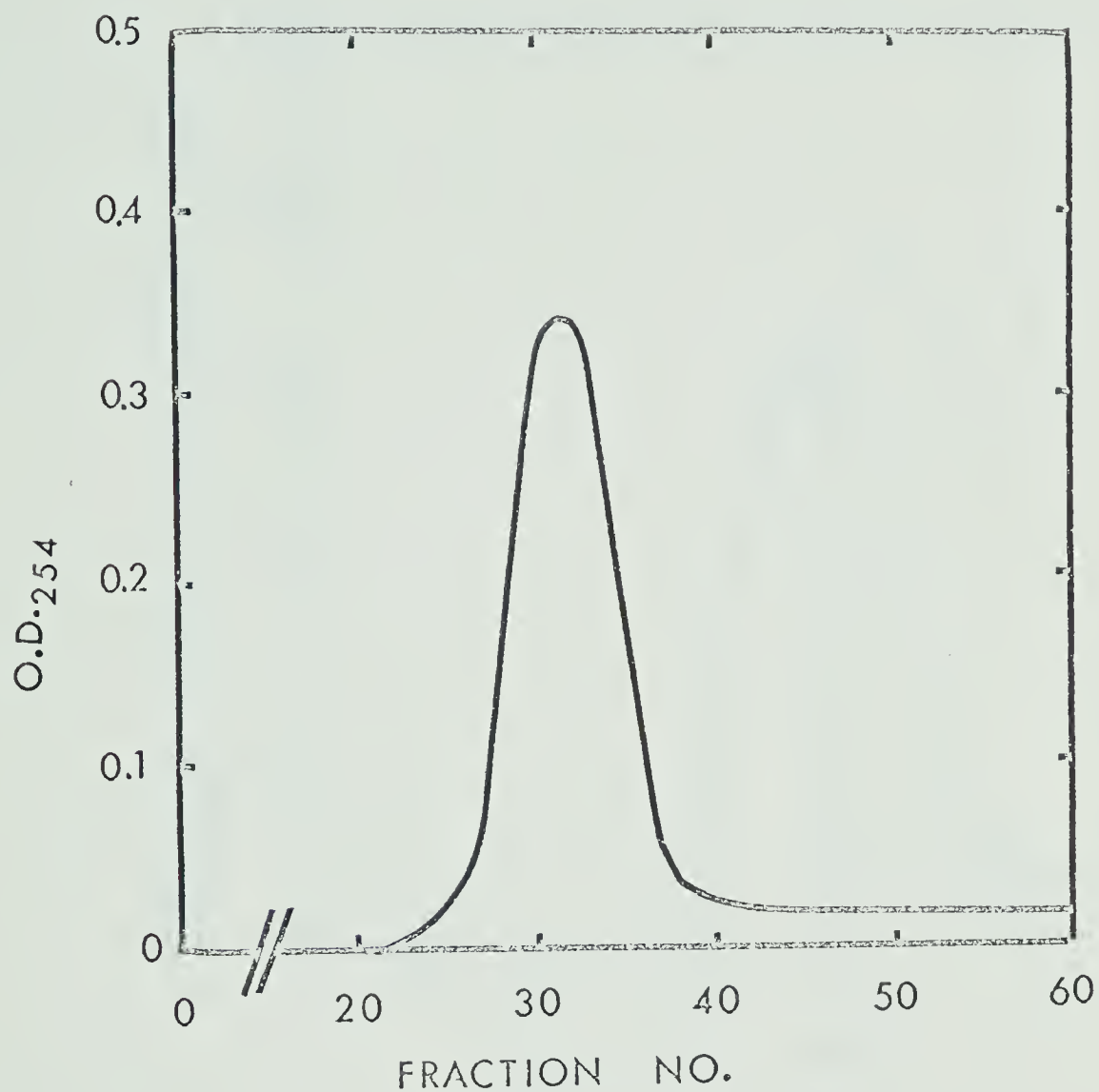


Figure 25: Chromatography of a papain digest of myosin on a Sephadex G-75 column. Applied 3 ml of concentrated digest to a 2.5 x 37 cm column pre-equilibrated with 0.05M KCl, 0.01M  $K_2HPO_4$  (pH 7.4), 1mM D.T.T., 4°C, with a descending elution of 2.6 ml/hr where 20 minute fractions were taken.



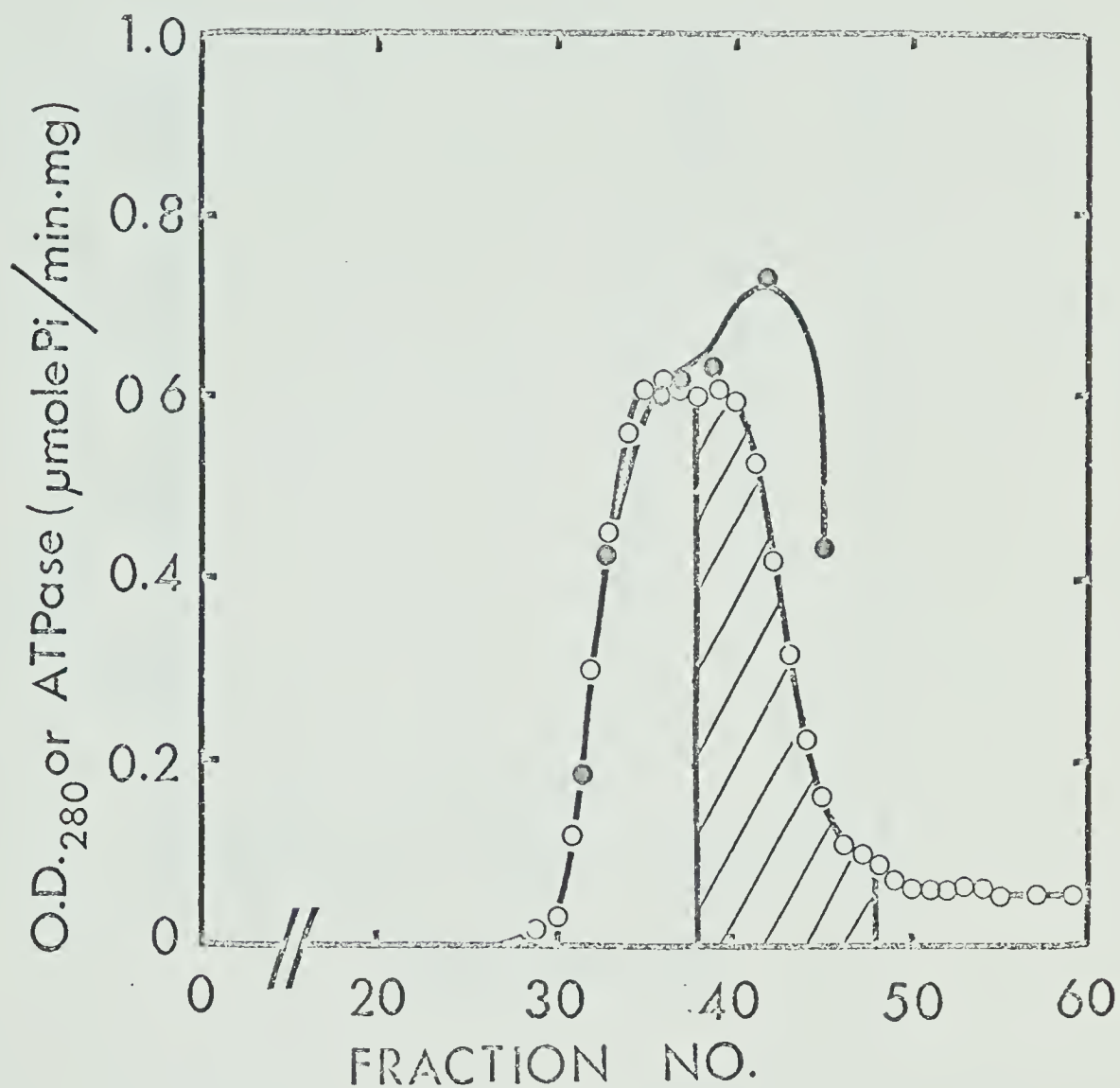


Figure 26: Chromatography of a papain digest of myosin on a Sephadex G-200 superfine column. Applied 3 ml of concentrated digest to a 2.5 x 30 cm column pre-equilibrated with 0.05M KCl, 0.01M  $K_2HPO_4$  (pH 7.4), 1mM D.T.T., 4°C, with a descending elution of 3 ml/hr where 1 hour fractions were taken. (O), O.D.<sub>280</sub> from which protein concentration was calculated using 770 cm<sup>2</sup>/g (Young, 1967b); (●), ATPase at 0.05M KCl, 0.05M tris-Cl (pH 7.4), 10mM  $CaCl_2$ , 1.0mM ATP, 25°C.



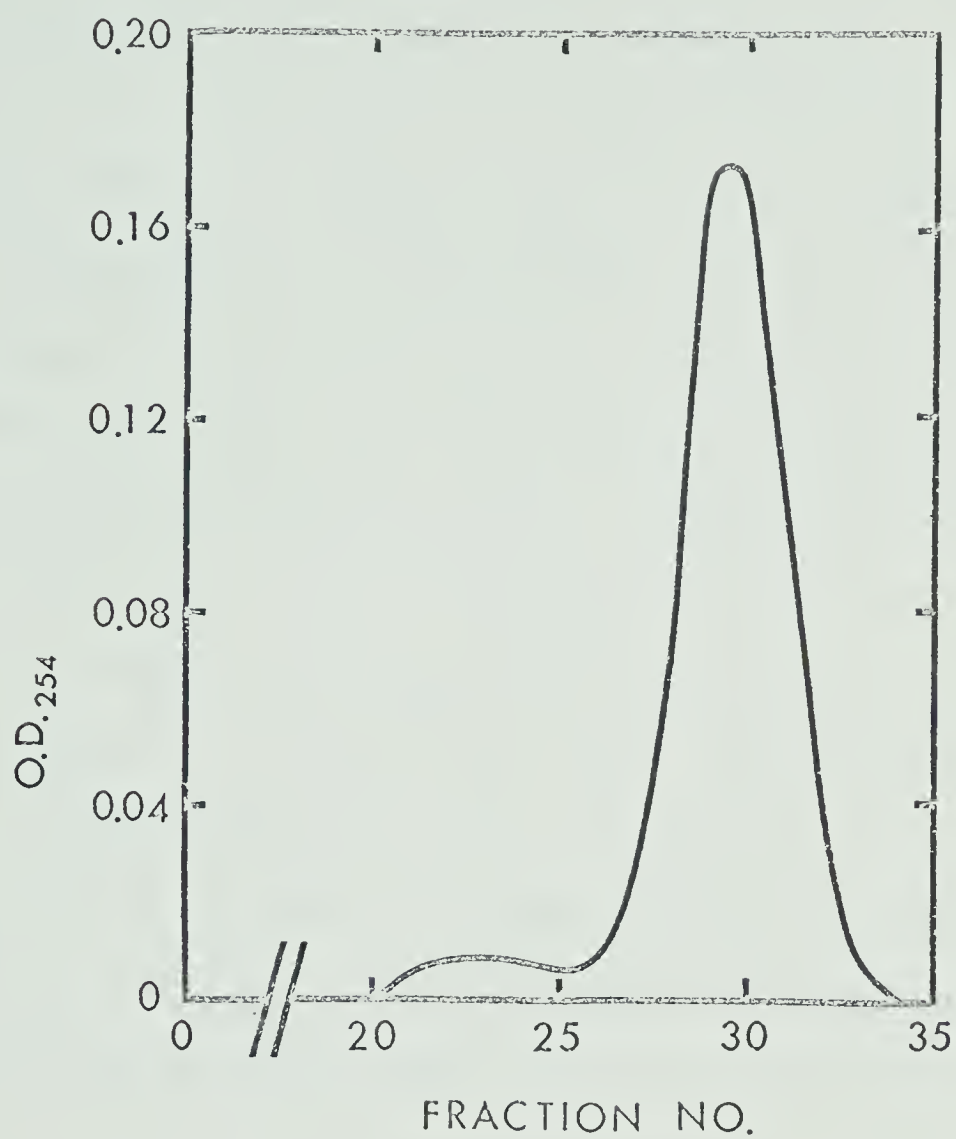


Figure 27: The second chromatography on Sephadex G-200 superfine. Applied the pooled fractions from Fig. 26 (hatched area) (3 ml) to a 2.5 x 30 cm column which was pre-equilibrated and eluted as in Fig. 26.



to detect a component in the supernatant material which possesses ATPase activity but is not capable of combining with actin. According to the results listed in Table 12, successive additions of F-actin to the pooled fractions of the main peak of Fig. 27 revealed that all the ATPase active material in these fractions was removed by F-actin.

The subfragment was subsequently dissociated from F-actin by Mg-pyrophosphate (MgPP) and dialysed against 0.5M KCl, 0.01M  $K_2HPO_4$  (pH 7.4), 1mM dithiothreitol (D.T.T.). Sedimentation studies revealed the presence of two components (Fig. 28, Plate I), but when the subfragment was treated with 0.1M  $\beta$ -mercaptoethanol, a single component was observed (Plate II).  $\beta$ -mercaptoethanol was found to equally effective at 0.02M. The observed S value of the single component in Plate II was found to be 5.0 at a protein concentration of 7.1 mg/ml and at 20°C which is in agreement with the results of Jones and Perry (1966) for active subfragment.

In the absence of more comprehensive data, the sedimentation of the various species observed in Plate I of Fig. 28 was not extensively investigated, but it seems likely that the more rapidly sedimenting peak could be due to an aggregate, such as a dimer, and that this aggregation is due to disulfide formation. This is also evident from the presence of heavier material in the chromatography of the subfragment prepared through the actin binding step (Fig. 29).

In order to determine the molecular weight of subfragment, a high-speed sedimentation-equilibrium analysis of samples treated with  $\beta$ -mercaptoethanol was carried out according to Yphantis (1964). For this experiment, the centrifugal speed was chosen such that the concentration at the meniscus was negligible in comparison to the initial concentra-





Table 12

Recovery of Subfragment as the Actin-Subfragment Complex

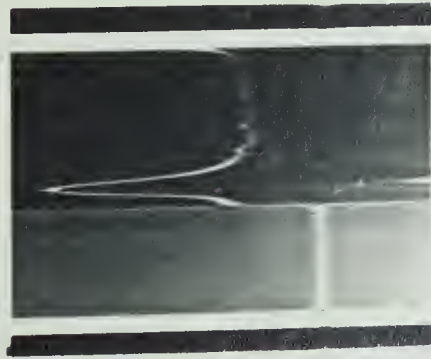
Centrifugation	ATPase ( $\mu$ mole Pi/min. mg)	Protein remaining in supernatant (mg/ml)
Initial	1.30	1.18
1	0.34	0.62
2	0.08	0.57

An aliquot (7.6 mg of 4.4 mg/ml) of F-actin was added to the pooled fractions of the main peak (Fig. 27) (14 mg). The addition of F-actin to the supernatant was repeated thrice. After each centrifugation at 120,000 x g for 1 hour, the  $\text{Ca}^{2+}$ -ATPase activity in the supernatant was determined at 0.05M KCl, 0.05M tris-Cl (pH 7.4), 10mM  $\text{CaCl}_2$ , 1.0mM ATP, 0.05-0.10 mg subfragment/ml, at 25°C.



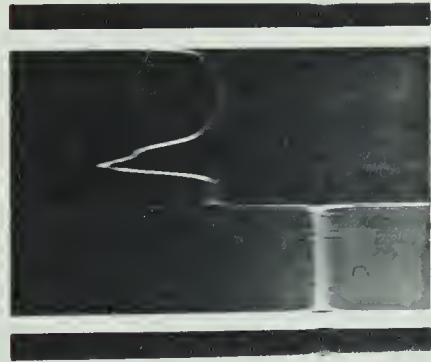
Figure 28: Sedimentation of the myosin subfragment. Conditions: 0.5M KCl, 0.01M  $K_2HPO_4$  (pH 7.4), 7.1 mg/ml, 20°C. Plate I, 1mM D.T.T.; Plate II, 0.1M  $\beta$ -mercaptoethanol. Kel F cell, bar angle is 55° for first picture and 50° for remaining pictures. Time after reaching full speed (59,780 rev./min.) is indicated under each frame.



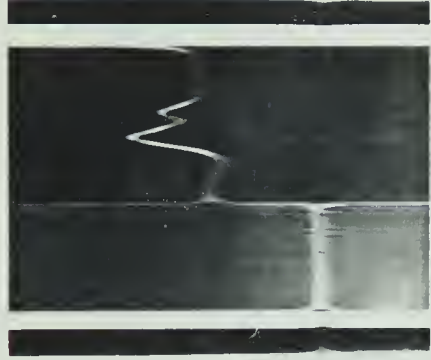


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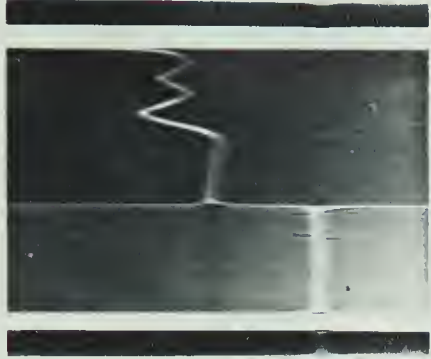
PLATE I



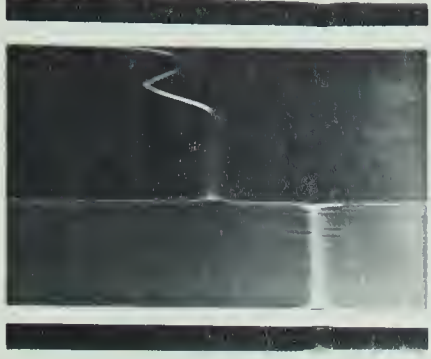
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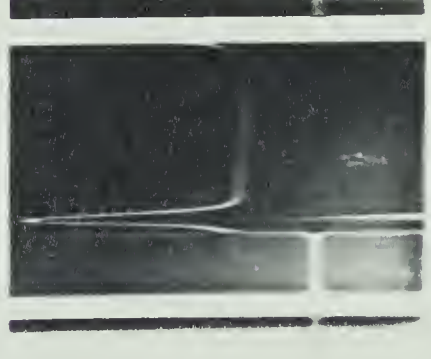
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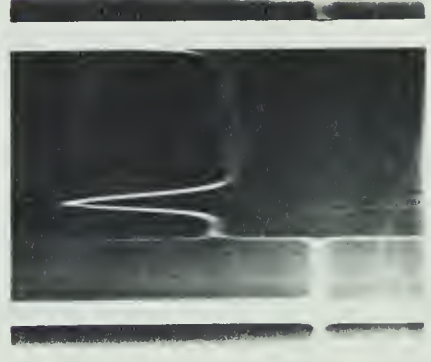


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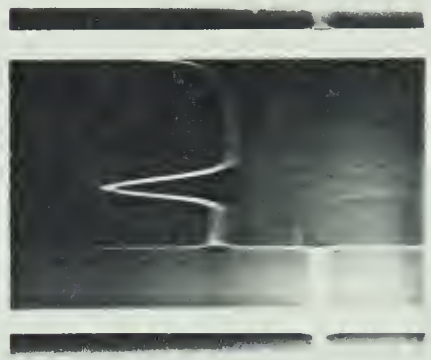


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PLATE II



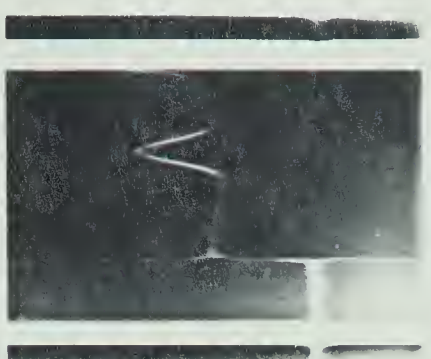
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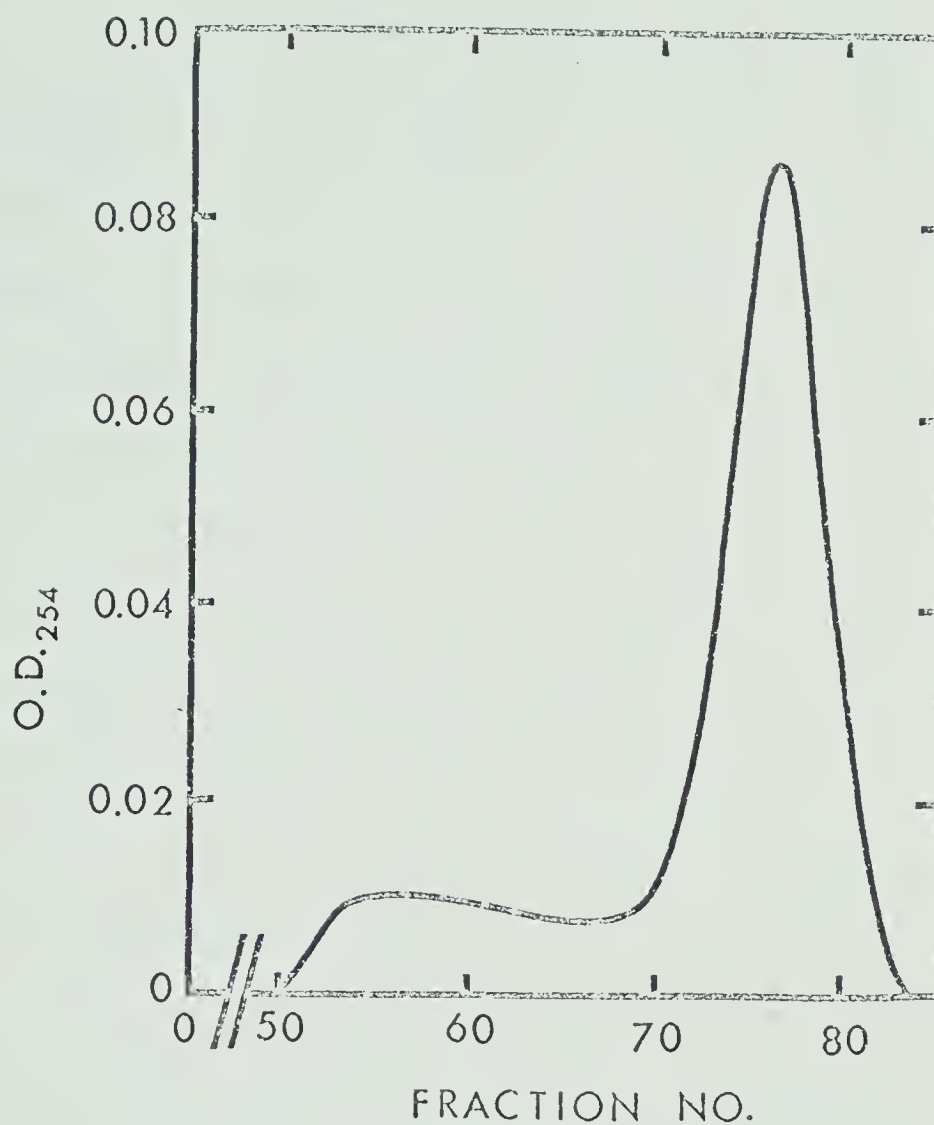


Figure 29. Chromatography of the actin-isolated myosin subfragment. An aliquot (3 ml) was applied to the top of a 2.5 x 30 cm column which was pre-equilibrated and eluted as in Fig. 26 except that 20 minute fractions were taken.





tion (see Fig. 5, Methods and Materials). In Fig. 30, a representative plot of the change in log (natural) fringe displacement is shown as a function of  $R^2$ , where  $R$  is the distance in cm from the center of the rotation to the position of the considered fringe displacement. At the highest protein concentration tested (1.8 mg/ml) a linear plot is obtained without detectable heterogeneity. There is no indication of a lighter species at or near the meniscus region. In all, five determinations of the weight average molecular weight were carried out on two different preparations of fragment (Table 13), assuming a partial specific volume of 0.74 ml/g (Mueller, 1965; Kominz et al., 1965). All the determinations, including those at different centrifugation times, show linear plots of log fringe displacement vs  $R^2$  with an equivalent molecular weight within experimental error, indicating that equilibrium was attained by 24 hours at 18,000 rev./min. Preparation No. 2 was digested for approximately twice the time as preparation No. 1 with no apparent change in the molecular weight. Similar findings by Lowey et al., (1969) support the idea that the subfragment molecule is somehow protected from further papain digestion. There is no observed concentration dependency within the range investigated, so that the values for weight average molecular weight (Yphantis, 1964) can be averaged to give a value of  $116,000 \pm 5,000$ , in excellent agreement with previous results (Nihei and Kay, 1968; Lowey et al., 1969; Tada et al., 1969). The findings reported here suggest that a prominent factor in the discrepancy for reported values of the subfragment molecular weight may be due to aggregation. Upon treatment with enough sulfhydryl reducing agent, the subfragment appears homogeneous within



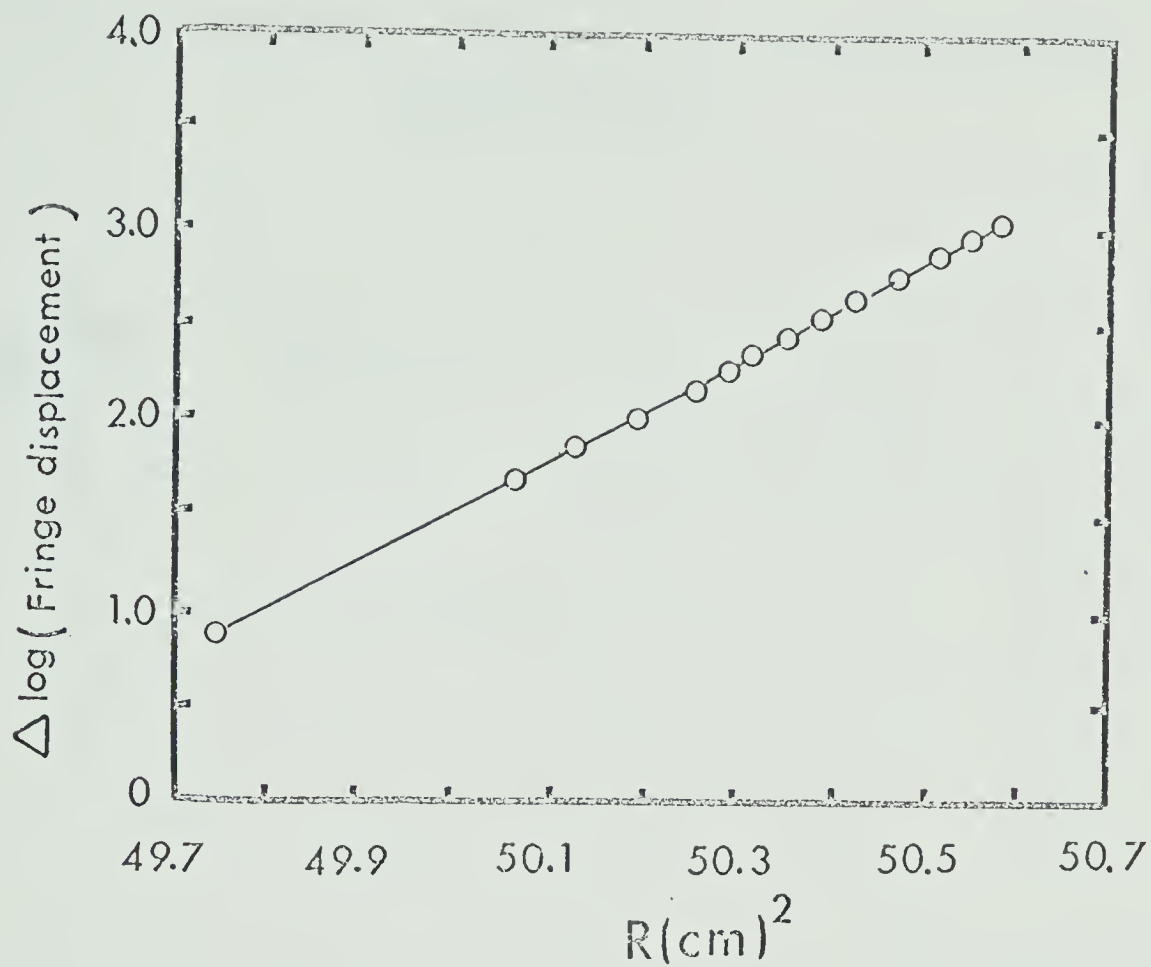


Figure 30. Sedimentation-equilibrium of the myosin subfragment. Conditions: 0.5M KCl, 0.01M  $\text{K}_2\text{HPO}_4$  (pH 7.4), 0.02M  $\beta$ -mercaptoethanol, 1.8 mg/ml. Run for 30 hours and 18,000 rev./min. at  $4^\circ\text{C}$ . The change in log fringe displacement (microns) was plotted as a function of  $R^2$ , the distance in cm from the center of rotation.



Table 13

The Sedimentation-Equilibrium of the Papain-Hydrolyzed  
Myosin Subfragment

Myosin Digest	Run No.	Digestion Time in minutes	Protein Conc. (mg/ml)	Time of run in hrs	Mw app
1	1	10	0.5	30	120,000
	2	(4H <sup>+</sup> /10 <sup>5</sup> gm myosin)	1.0	24	110,000
	3		1.8	30	120,000
	4		1.8	46	120,000
2	5	19-27	0.9	30	110,000
					116,000±5,000

The preparations were made by the centrifugation of the actin plus myosin digest mixture. Conditions: 0.5M KCl, 0.01M K<sub>2</sub>HPO<sub>4</sub> (pH 7.4), 4°C. All samples contained 0.02M β-mercaptoethanol. Density of solvent was 1.025 g/ml determined from the contribution of each dissolved species.



the range of experimental error shown in Table 13 although it cannot be ruled out that some heterogeneity exists due to an incomplete digestion or an internal proteolysis.

Table 14 depicts the increase in  $\text{Ca}^{2+}$ -ATPase at the various stages of subfragment preparation. Despite the considerable evidence of two ATPase active sites on myosin (see Introduction), the specific activity at subfragment is 3-4 times that of myosin, which is about twice as high as expected. This discrepancy may be due to the sulfhydryl reducing agents  $\beta$ -mercaptoethanol and D.T.T. used in the preparation of subfragment, which may increase the  $\text{Ca}^{2+}$  or  $\text{Mg}^{2+}$  activated ATPase in the same way that pMB or NEM does (Kielly and Bradley, 1956; Perry and Cotterill, 1965; see also Discussion).





Table 14

The  $\text{Ca}^{2+}$ -ATPase of Myosin Subfragment

Sample	ATPase ( $\mu\text{mole Pi/min. mg.}$ )		
	1	2	3
Myosin	0.44	0.47	0.41
Crude digest after separation of fraction insoluble at 0.05M KCl	0.95		
Pooled fractions from first chromatogram (Hatched area Fig. 26)	1.30		
Actin isolated by centrifugation of an actin subfragment complex		1.62	1.65
Ratio of the activity of subfragment to myosin		3.4	4.0

Determined at successive stages in the preparation at 0.05M KCl, 0.05M tris-Cl (pH 7.4), 10mM  $\text{CaCl}_2$ , 1.0mM ATP, 0.12 mg myosin/ml, 0.05-0.10 mg/ml subfragment/ml at 25°C.



#### 4. The Interactions of F-actin with Myosin and Subfragment

From the available information to date, the interaction between actin and myosin is considered as the molecular basis of muscle contraction. In this interaction, a part of myosin that can be separated as heavy meromyosin from the rest of the molecule makes contact with actin. It has been shown by many investigators that there are two sets of sites capable of binding with actin and catalyzing ATP hydrolysis in one unit of heavy meromyosin. The subfragment of myosin prepared through papain digestion possesses these sites. The detailed information concerning the interaction of this subfragment and actin was thought to give some insight into the mechanism of the actin-myosin interaction.

Previously, the affinity of actin to myosin was estimated assuming that the extent of the complex formation is proportional to the increment of viscosity or turbidity of the mixture of the two proteins. However, it was found not to be possible to measure the binding reaction at low concentrations of KCl ( $<0.1M$  KCl) since actomyosin becomes a hydrated gel capable of superprecipitation and the viscosity or turbidity increase associated with this process is distinct from that due to actomyosin formation.

An early investigation established the close relationship between the actin combining property of myosin and the ATPase activity (Bailey and Perry, 1947). It is known that the addition of F-actin to myosin results in an increase in the  $Mg^{2+}$ -ATPase at low concentrations of KCl ( $<0.1M$ ). It is thus possible to use this property as a criterion of actin-myosin binding. This requires a consideration of the equilibria



for a mixture of F-actin, myosin  $Mg^{2+}$  and ATP. Accordingly, Botts and Morales (1953) and Botts (1958) formulated a kinetic scheme considering the actomyosin formation in the presence of a large molar excess of substrate.

Both KCl and ATP tend to dissociate F-actin from myosin, heavy meromyosin, or subfragment as reflected by a viscosity decrease or a decrease in the  $Mg^{2+}$ -ATPase activity (Gergely, 1956; Eisenberg and Moos, 1967; Eisenberg et al, 1968; Tawada and Oosawa, 1969; Rizzino et al., 1970). The experiments for the binding reaction between F-actin and myosin or subfragment have been conducted at low concentration of KCl and at high levels of ATP such that there would be almost complete substrate saturation of the myosin and actomyosin enzyme systems. The concentration of 1.0mM ATP used for the present analyses of the  $Mg^{2+}$ -ATPase of a mixture of actin and myosin is twenty times as high as the  $K_m$  value for  $Mg^{2+}$ -ATPase (0.05mM, Fig. 21). For the measurement of activity in an actin-subfragment system the concentration of ATP was 0.5mM (Eisenberg et al., 1968; Szentkiralyi and Oplatka, 1969). This concentration seemed to saturate the enzyme with ATP since at increased concentration, e.g. 2.0mM, the results were the same as that at 0.5mM ATP (Eisenberg et al., 1968). An excess of  $MgCl_2$  was added such that most of ATP was in the form of  $MgATP^{2-}$ , and consequently there was sufficient concentration of free  $Mg^{2+}$  to give maximum activation of the actomyosin and actin-subfragment ATPase.

The rate of ATP hydrolysis,  $v$ , catalyzed by a mixture of myosin and actomyosin which are saturated by ATP, can be expressed by the



following equation:

$$v = k_3\{M\} + k_3'\{AM\}$$

where  $k_3$  and  $k_3'$  are the rate constants of the step at which the enzyme-substrate complex forms the products, and the concentrations of myosin and actomyosin are represented by  $\{M\}$  and  $\{AM\}$ , respectively. This equation may also be written as:

$$v = k_3 (\{Mo\} - \{AM\}) + k_3' \{AM\}$$

where  $\{Mo\} = \{M\} + \{AM\}$ .

In order to test the binding reaction, the  $Mg^{2+}$ -ATPase activity was determined in various mixtures of F-actin and myosin: (1) different concentrations of F-actin were added to a constant concentration of myosin, and (2) the concentration of F-actin was held constant while myosin was varied. These two sets of data can be used to estimate the number of actin binding sites per mole of myosin.

#### A. Actin-Myosin Binding

In Fig. 31, the  $Mg^{2+}$ -ATPase activity was plotted against the ratio of F-actin and myosin. Under the experimental condition tested, no ATPase activity was detected in F-actin. The ratio of F-actin to myosin in Fig. 31a was calculated on the basis of a molecular weight of 500,000 daltons for myosin (Tonomura et al., 1966) and of 60,000 daltons for actin monomer (Kay, 1960; Lewis et al., 1963; Krans et al., 1965). Recently, the molecular weight of chromatographically purified actin showed a value of 46,000 daltons (Rees and Young, 1967), which does not, however, alter the order of magnitude of the estimated binding constant for the actin-myosin interaction (Fig. 31b).





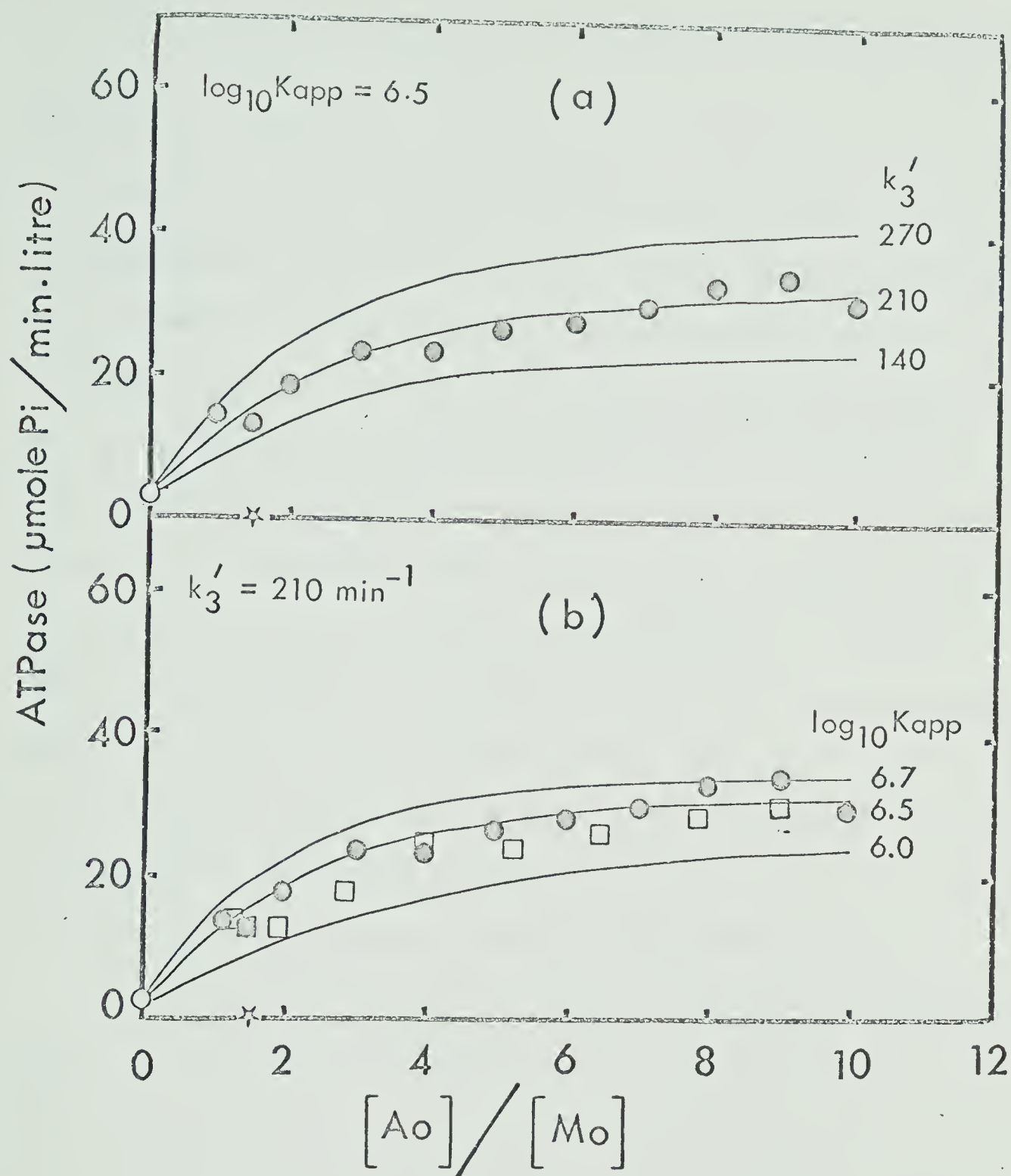


Figure 31: The relationship between  $\text{Mg}^{2+}$ -ATPase and the actin to myosin ratio. The reaction mixture contained 0.010M KCl, 0.02M tris-Cl (pH 7.4), 5.0mM  $\text{MgCl}_2$ , 1.0mM ATP, 0.090 mg myosin/ml. The reaction at 25°C was started by adding ATP and aliquots were taken for Pi measurements at time intervals of 15 seconds. The values for  $v$  were computed assuming a mole per mole association with actin. The values of the constants used for the calculations are described in the text. (O), myosin alone; (☆), F-actin alone; (●) and (□), F-actin plus myosin assuming an actin M.W. of 60,000 and 46,000 daltons, respectively.



It is evident that there are two ATPase sites per myosin molecule. In view of the role of actin-myosin interaction in the contractile phenomenon, it is important to see whether or not both of these sites can be modified independently. The experimental results, however, were consistent with two assumptions: (1) only one of the ATPase sites in myosin can be modified with actin thereby giving rise to the increased  $Mg^{2+}$ -ATPase activity; and (2) the binding of one mole of actin to one mole of myosin affects both sites simultaneously. This was also shown with heavy meromyosin, which possesses two potential ATPase sites (Szentkiralyi and Oplatka, 1969; Rizzino et al., 1970). If one mole of actin bound to myosin results in the modification of only one of two sites, leaving the other site free, this free site should be considered to give the ATPase activity of free myosin. Since the activity of free myosin in the presence of  $Mg^{2+}$  is very low (see Table 15), the consideration of a remaining free ATPase site did not give a detectable difference in the calculated curves from those shown in Fig. 31.

For the calculations, the formation of actomyosin for different values of the apparent association constant was computed as a function of the actin to myosin ratio, where it was assumed that one actin binds to each molecule of myosin (Fig. 32). The assumed values of  $K_{app}$  for actomyosin formation were then entered into the equation for  $v$ , and using an APL program, were used to calculate a set of curves corresponding to various values of  $k'_3$ .

At  $\log_{10} K_{app}$  value of 6.5, the actomyosin formation approximates the experimental data for a value of  $k'_3$  equal to  $210 \text{ min}^{-1}$  as shown in Fig. 31a, and from Fig. 31b, it is apparent that other values of



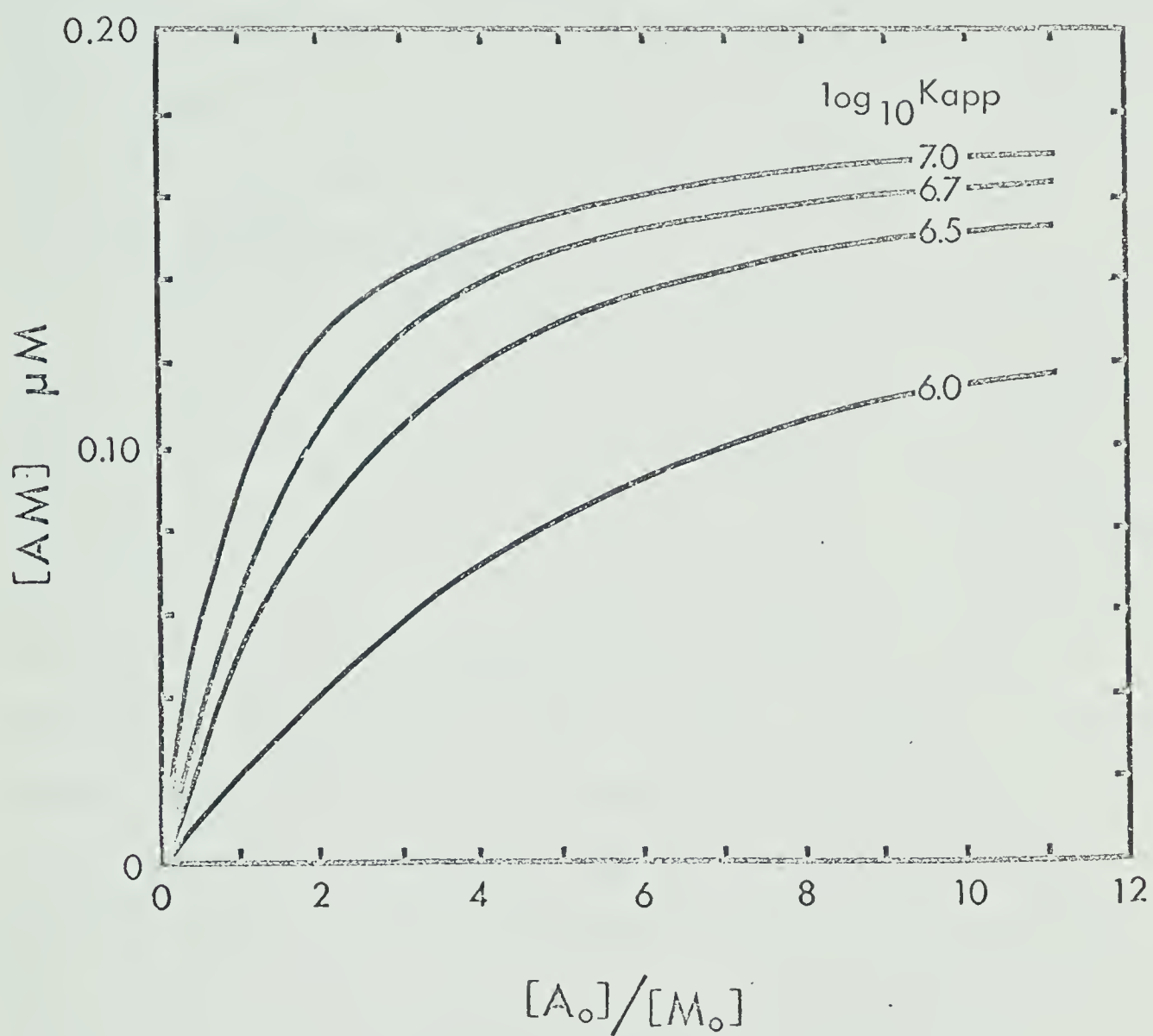


Figure 32: Computed formation of actomyosin where 0.18  $\mu M$  myosin is available for actin combination.



$\log_{10} K_{app}$  would not correspond as well to the data.

The increase in the activity when a varied amount of myosin is added to actin is shown in Fig. 33. The activity due to myosin alone was found to be proportional to the myosin concentration within the range investigated. Theoretical curves for the velocity of the  $Mg^{2+}$ -ATPase were computed as above where a  $\log_{10} K_{app}$  of 6.7 and  $k_3'$  of  $270 \text{ min}^{-1}$  were found to agree with the experimental results.

#### B. Actin-Subfragment Binding

The above relationships between the increase in the  $Mg^{2+}$ -ATPase and the formation of actomyosin may also be used for analysing the binding reaction between actin and the subfragment where actin is expected to affect the single ATPase site of each subfragment (Young, 1967a and b; Eisenberg et al., 1968). In Fig. 34, the  $Mg^{2+}$ -ATPase activity is also seen to increase with the addition of actin to subfragment, except that it is apparent that the binding is much weaker. These data were plotted assuming a molecular weight of 116,000 daltons for subfragment. Fig. 34b indicates also that a molecular weight of 46,000 daltons for actin does not appreciably alter the plot. Various values of  $k_3'$  at a  $\log_{10} K_{app}$  value of  $160 \text{ min}^{-1}$  approximates the data most closely. However, the experiments were not able to show the exact change in slope with increasing levels of added F-actin. As a result, there is no reason to exclude the possibility that the actin-subfragment binding constant is lower (Fig. 34b) with a corresponding higher value of  $k_3'$  being required to fit the data. It is unlikely however, that the binding exceeds  $2 \times 10^4 M^{-1}$ , since a higher association constant of complex formation would show





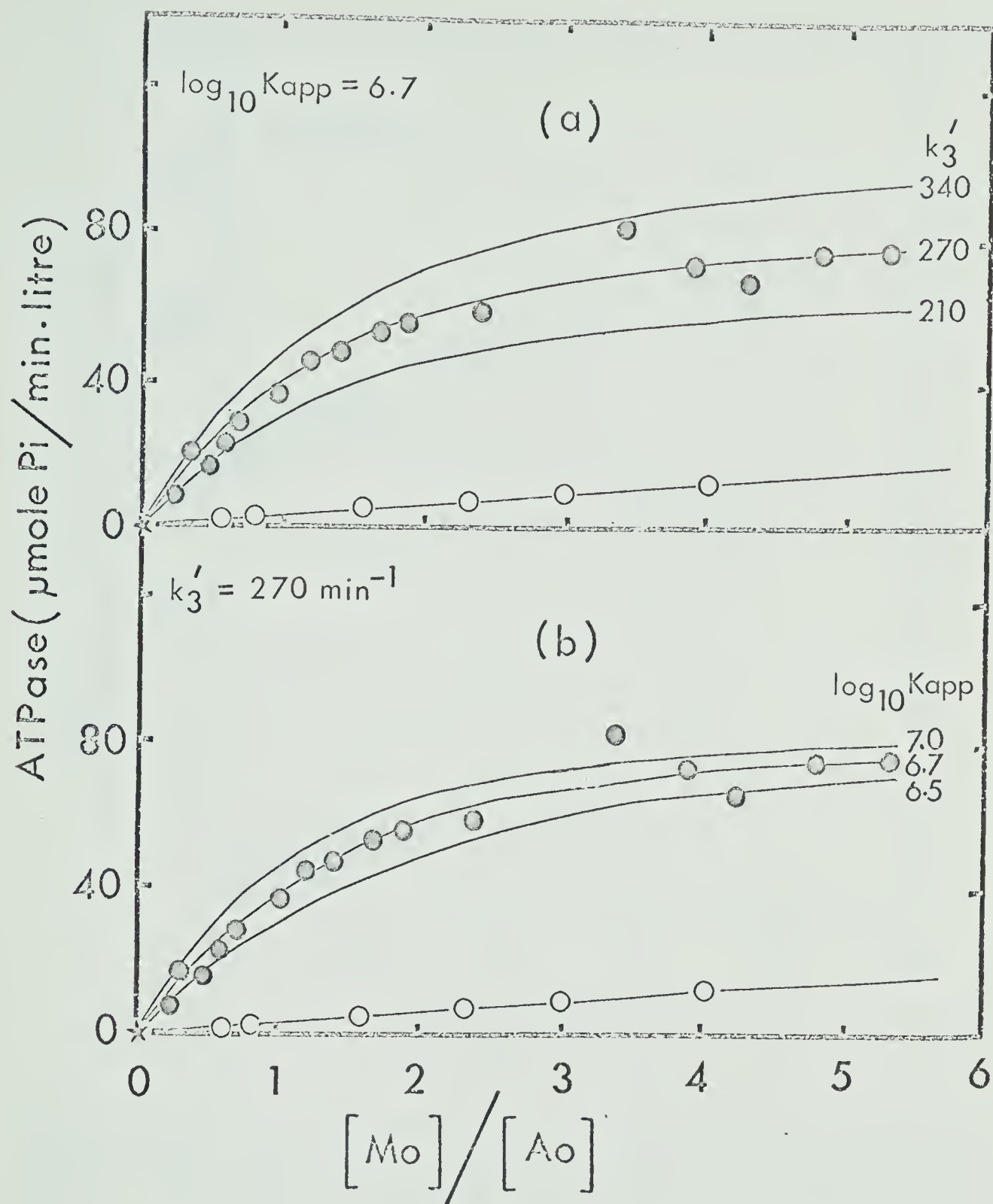


Figure 33: The relationship between Mg<sup>2+</sup>-ATPase and the myosin to actin ratio. The reaction mixture contained 0.008-0.024M KCl, 0.02M tris-Cl (pH 7.4), 5.0mM MgCl<sub>2</sub>, 1.0mM ATP, 1.0mM EDTA, 0.018 mg F-actin/ml. The reaction at 25°C was started by adding ATP and aliquots were taken for Pi measurements at time intervals of 15 seconds. The values for  $v$  were computed as in Fig. 31, and the symbols are also the same. The values of the constants used for the calculations are described in the text.



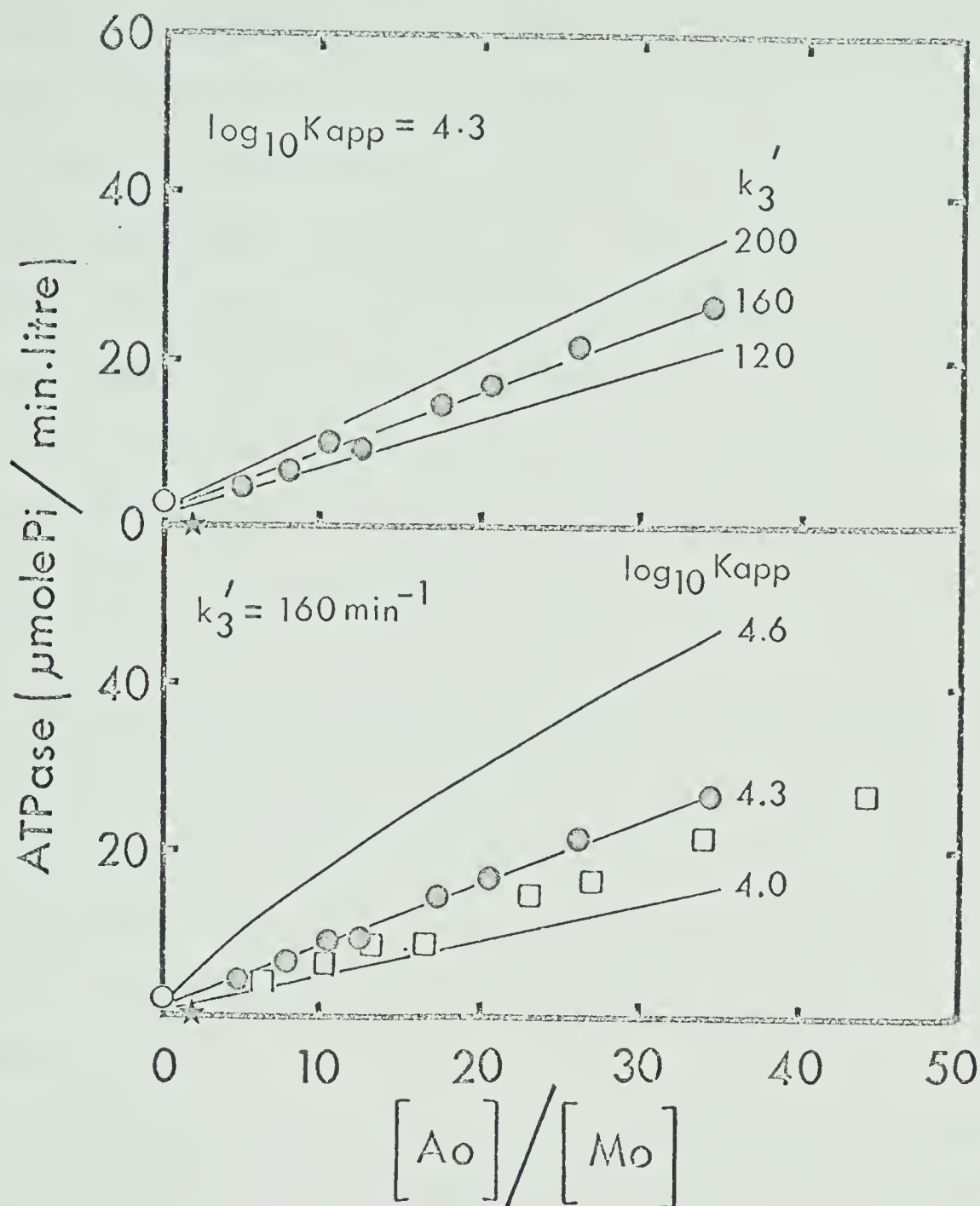


Figure 34: The relationship between the  $\text{Mg}^{2+}$ -ATPase and the actin to subfragment ratio. The reaction mixture contained 0.025M KCl, 0.02M tris-Cl (pH 7.4), 1.0mM  $\text{MgCl}_2$ , 0.5mM ATP, 0.032 mg subfragment/ml. The reaction at 25°C was started by adding ATP and aliquots were taken for Pi measurements at time intervals of 1 minute. The values for  $v$  were computed assuming that there is one active site per mole of subfragment which combines with actin. The values of the constants used for the calculations are described in the text. (O), subfragment alone; (☆), F-actin alone; (●) and (□), F-actin plus myosin assuming an actin M.W. of 60,000 and 46,000 daltons, respectively.



a curvature incompatible with the data. Other authors have claimed to have determined the exact strength for the binding reaction within the same range of actin or subfragment concentrations used in these experiments (Eisenberg et al., 1968; Lowey et al., 1969). However, their use of a reciprocal plot is questioned because the slight changes of the ordinate intercept would show large variations in the value of the maximum velocity and the value for the free concentration of F-actin required for the calculation would not be equal to the total concentration of actin used for the reciprocal plot.

The corresponding experimental results for the addition of subfragment to F-actin were found to agree quite well with the values of the association constant and  $k_3'$  for F-actin addition to subfragment (Fig. 35). It is also shown that the  $Mg^{2+}$ -ATPase activity of subfragment alone is proportional to the subfragment concentration.

The values of  $K_{app}$  and  $k_3'$  which are appropriate to explain the experimental data are summarized in Table 15. The good agreement between the two sets of values for the binding reaction between F-actin and myosin or F-actin and subfragment is compatible with a mole per mole association in either case.

Assuming that the two sites in myosin bind with two actin monomers would involve introducing unrealistic values of  $k_3'$ , whereas it doesn't seem likely that only one site in myosin can react with actin. Rather, it may be speculated that because of the large size of the actin monomer ( $50\text{\AA}$ ) and its arrangement in the double-



Figure 35: The relationship between the  $\text{Mg}^{2+}$ -ATPase and the subfragment to actin ratio. The reaction mixture contained 0.025M KCl, 0.02M tris-Cl (pH 7.4), 1.0mM  $\text{MgCl}_2$ , 0.5mM ATP, 0.017 mg F-actin/ml. The reaction at 25°C was started by adding ATP and aliquots were taken for  $\text{P}_i$  measurements at time intervals of 1 minute. The values of  $v$  were computed as in Fig. 34, and the symbols are also the same. The values of the constants used for the calculations are described in the text.





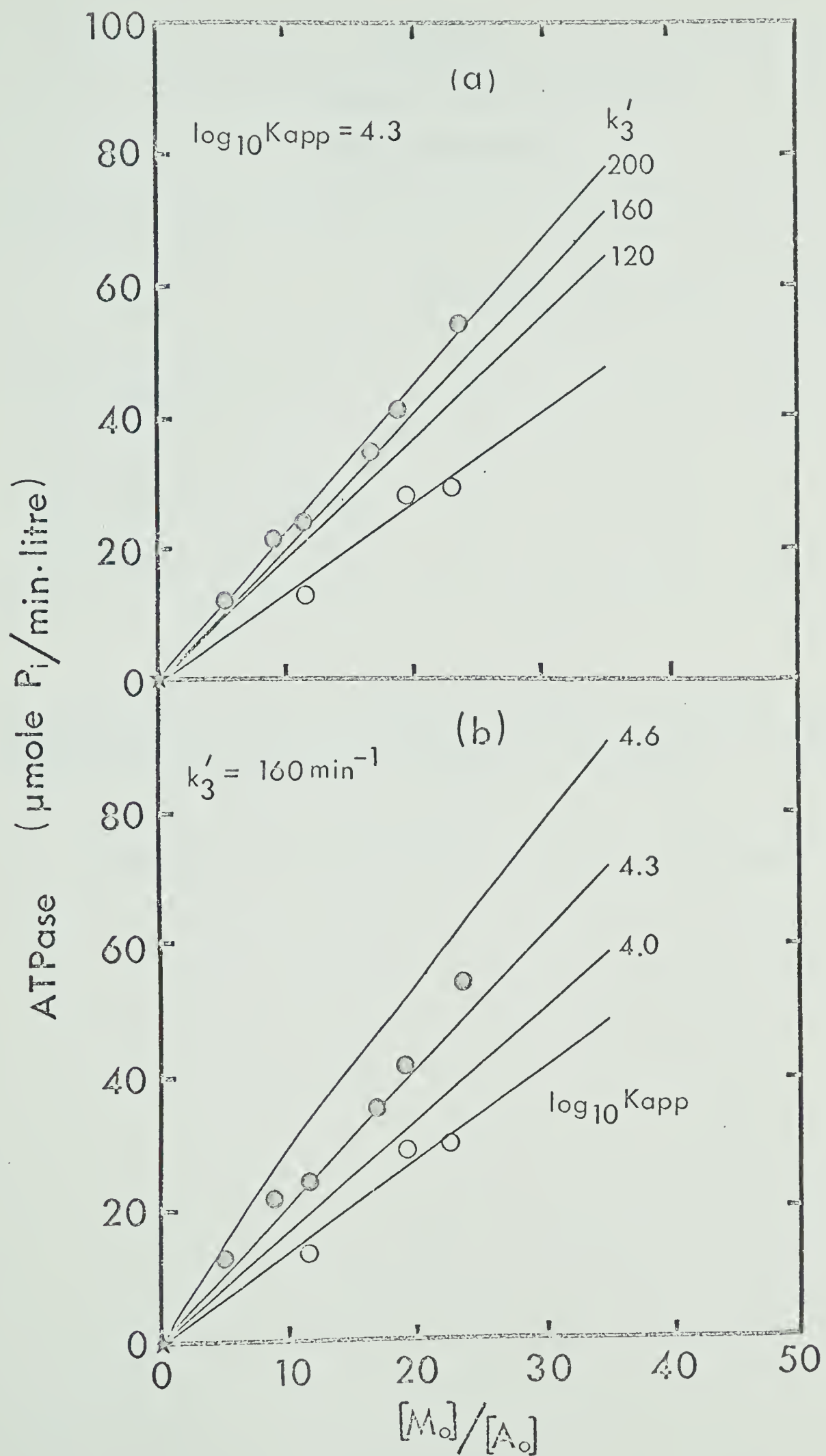




Table 15

The Actin-Myosin and Actin-Subfragment

Binding Parameters

	$\log_{10} K_{app}$	$k_3' \text{ (min}^{-1}\text{)}$	$k_3 \text{ (min}^{-1}\text{)}$
Actin added to myosin	6.5	210	5.2*
Myosin added to actin	6.7	270	---
Actin added to subfragment	4.3	160	4.5*
Subfragment added to actin	4.3	160	---

The values of  $k_3$  and  $k_3'$  represent the rate constants for the breakdown of the enzyme-substrate complex for free myosin or subfragment and the corresponding actin-complexes, respectively.

\* - determined from the  $Mg^{2+}$ -ATPase of myosin or subfragment



stranded, helically-wound F-actin polymer (Hanson and Lowy, 1963), and due to the close arrangement of the myosin sites in the 50<sup>0</sup>Å wide head portion of myosin, a binding of one actin monomer would produce a steric hindrance to the second binding site.



## 5. A Study of the Actin-Subfragment Interaction by Light Scattering

It was reported recently that in addition to the turbidity and volume changes of actomyosin gel (Levy and Fleisher, 1965; Sekine and Yamaguchi, 1966; Nihei, 1967), the polarization ratio of light scattered by the gel suspension can be used as an indicator phenomenon of the superprecipitation process (Nihei and Yamamoto, 1969). Hence, it seemed of interest to examine whether or not a change in the light scattered by a mixture of F-actin and the subfragment of myosin can be detected under the conditions which induce superprecipitation.

For this purpose, it was important to establish that actin was in the polymerized (F-actin) state under the conditions of the experiment. As reported by Oosawa et al., (1959), G-actin polymerizes only above a certain critical concentration of  $K^+$ ,  $Mg^{2+}$ , and ATP. Above the critical concentration, however, the ratio of F-actin to G-actin at equilibrium is independent of protein concentration.

For an understanding of the following light scattering experiments, it is necessary to consider certain basic principles. The angular distribution of scattered light is expressed as,

$$\frac{Kc}{I\theta} = \frac{1}{MP(\theta)} + 2BcP(\theta)$$

where  $I\theta$  is the Rayleigh Ratio of the scattering intensity,  $M$  is the molecular weight,  $P(\theta)$  is the interference factor accounting for the interaction of radiations from different parts of the molecule,  $B$  is the protein interaction term and  $c$  is the protein concentration.

Using the optical constants included in  $K$ , it is possible, by plotting





$Kc/I\theta$  versus  $\sin^2(\theta/2)$  and extrapolating to zero protein concentration and zero angle, to quantitatively evaluate the above parameters (Zimm, 1948). The molecular weight could thus be determined, since, with this extrapolation,  $B$  approaches zero and  $P(\theta)$  becomes unity. The magnitude of the slope term at various angles in the Zimm plot is proportional to the  $P(\theta)$  factor, which depends upon the size and shape of the scattering particles. In addition, any change in this slope can signify heterogeneity, since there is a greater contribution to the scattering from large particles at low angles.

A large polymer such as F-actin would thus have a large  $P(\theta)$  factor and give a high scattering intensity at low angles. The Zimm plot of Fig. 36 is observed to show a steep slope indicating the presence of F-actin (these light scattering intensities were measured with vertically and horizontally polarized light at various angles and a sum of the intensities at each angle was used to express the intensity of unpolarized light plotted in Fig. 36 and Fig. 37). It is predicted that a depolymerization of F-actin to G-actin monomers would result in a significant change in the slopes of Fig. 36 due to proportionally less scattering at low angles. However, the slopes are not different within the range of protein concentrations used, suggesting that actin remains in the polymerized state under the experimental conditions. The change in slope between  $60-90^\circ$  indicates that large size particles, which should be treated according to the Mie (1908) theory, contribute to the scattering. The polydispersity here becomes more obvious at higher protein concentrations.

For the study of the angular scattering of actin-subfragment



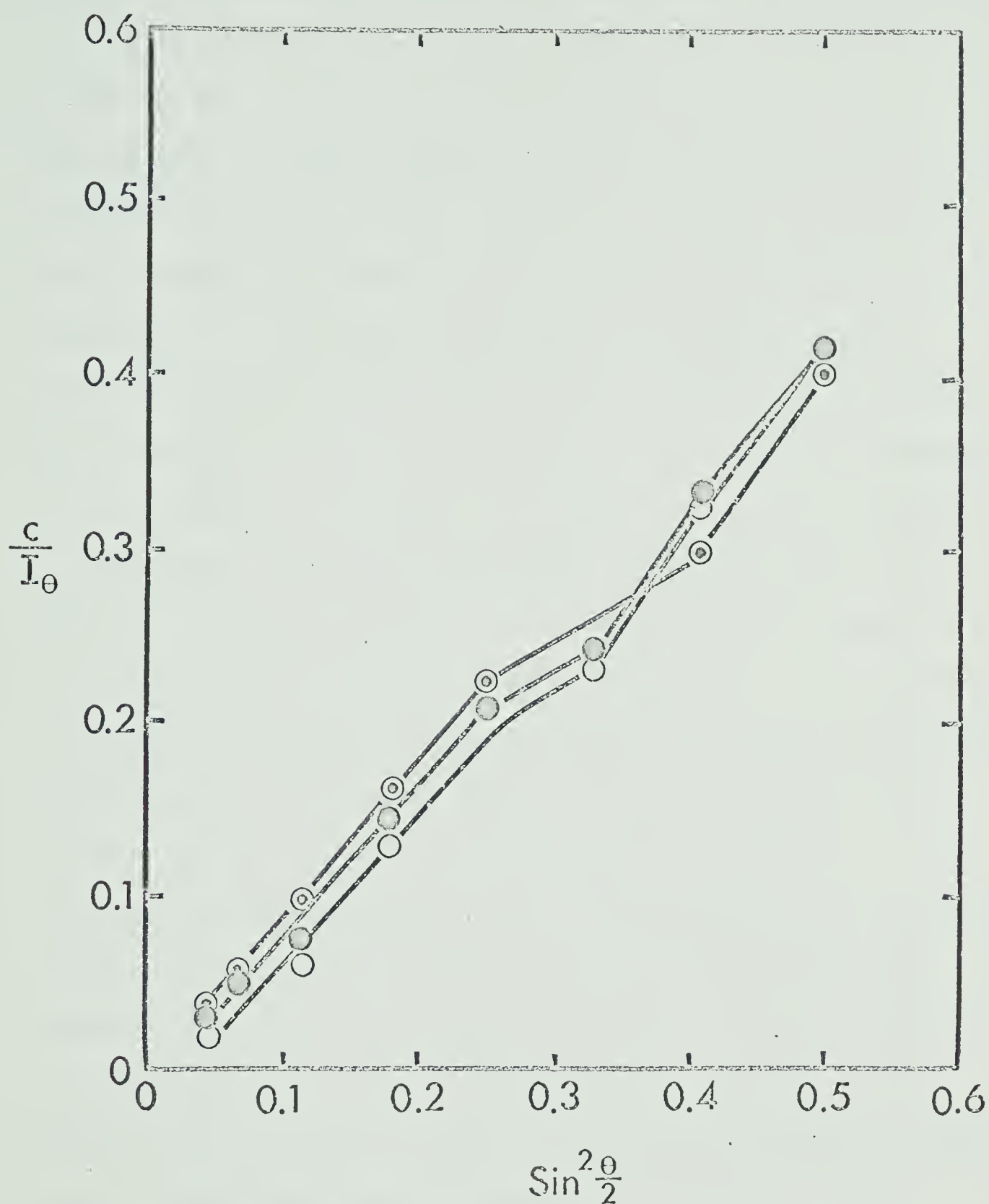


Figure 36: Zimm plot of F-actin light scattering. Various concentrations of F-actin were tested at 1.0mM  $\text{MgCl}_2$ , 0.1mM ATP, 0.01M tris-Cl (pH 7.4), 25-30°C. (○), 0.140 mg/ml; (●), 0.300 mg/ml; (⊙), 0.525 mg/ml.



complex, the scattering intensities (unpolarized light) were plotted as a function of the scattering angle (Fig. 37). A single preparation of F-actin was used for this analysis because it seemed likely that changes in the size distribution of the F-actin polymers would affect scattering to a large degree. A solution of F-actin at 0.17 mg/ml was centrifuged at 20,000 r.p.m. for 20 minutes in a Sorvall rotor to remove dust. This solution was stirred by a magnetic stirrer fitted under the scattering cell, and the measurement was repeated. Fig. 37 shows the light scattering from F-actin which is independent of stirring even at low angles. An aliquot of the stock solution of subfragment (0.05M KCl, 0.01M  $K_2HPO_4$ , pH 7.4 1mM D.T.T., 4.7 mg/ml) was added with stirring to a solution of F-actin until the final concentration reached 0.26 mg subfragment/ml. The concentration of F-actin in this solution was 0.16 mg/ml, which gave an approximately equimolar ratio of the subfragment to actin. The observed 3-fold increase in the scattering intensity at  $30^\circ$ , compared to an 8-fold increase at  $130^\circ$ , was expected since the small spherical subfragment molecule would not show the high scattering at low angles where F-actin dominates the scattering. For the measurement of light scattering in the presence of 0.1mM ATP, the solution was stirred for 10 minutes after the addition of ATP. Under this condition the ATPase activity was found to be 0.33  $\mu$  mole Pi/min.mg compared to an activity of 0.19  $\mu$  mole Pi/min.mg for the subfragment alone. After 10 minutes at this rate of hydrolysis, ATP should be virtually exhausted. The scattering intensities were determined and plotted as unpolarized light at the various angles but no change was detected



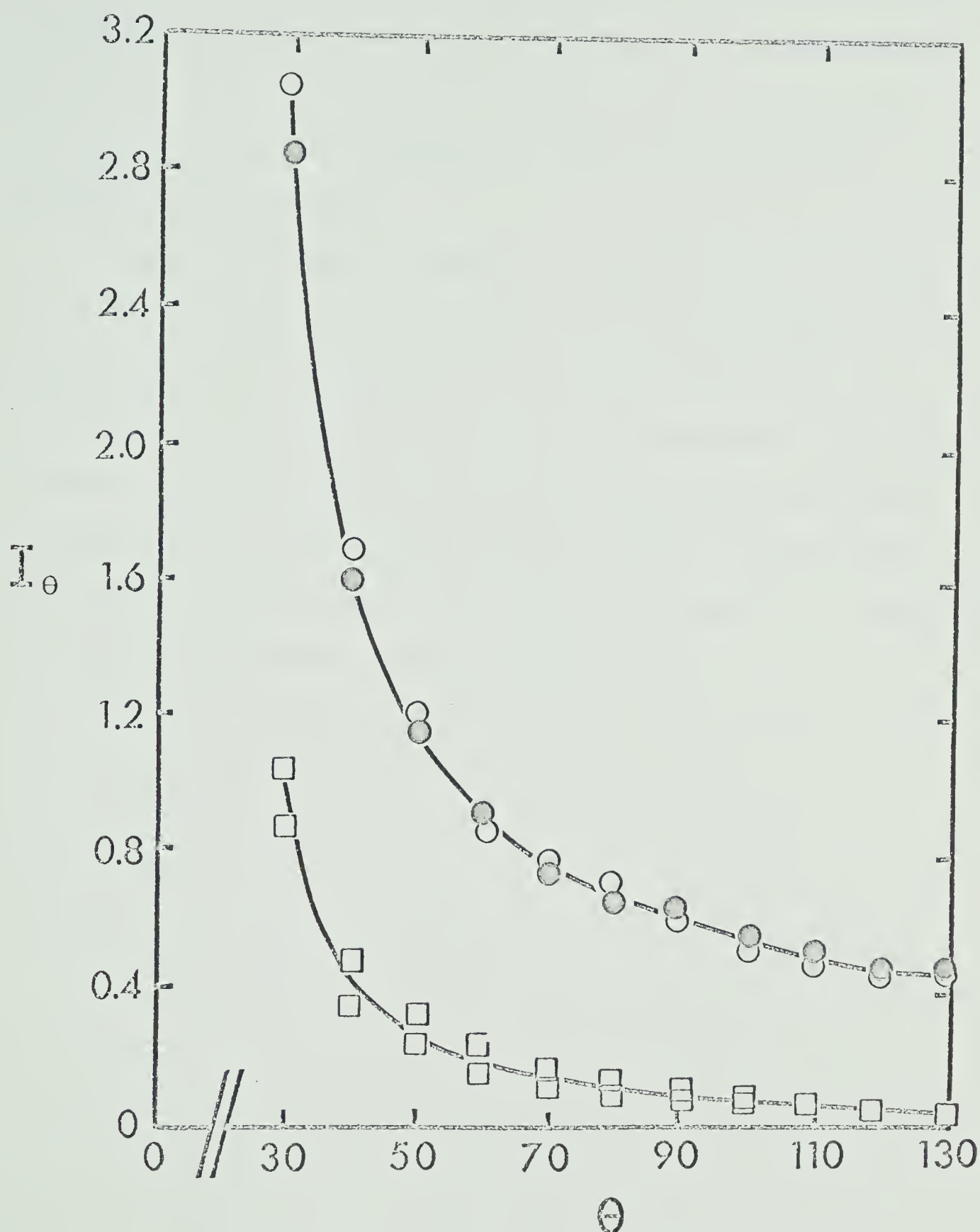


Figure 37: Light scattering intensity from solutions of F-actin and actin-subfragment mixtures. 1.0mM  $\text{MgCl}_2$ , 0.01M tris-Cl (pH 7.4), 25-30°C. ( $\square$ ), duplicates of F-actin scattering at 0.17 mg/ml; ( $\circ$ ), 0.16 mg/ml of F-actin plus 0.26 mg/ml of myosin subfragment; ( $\bullet$ ), this same actin-subfragment mixture after reaction with 0.1mM ATP.





in this or in either of the polarized components. The subfragment binding to F-actin was examined by centrifuging the actin-subfragment mixture at 110,000x g for 1 hour. The supernatant contained only 0.14 mg protein/ml, i.e. approximately 50% of the subfragment remained bound to F-actin.

As observed by light scattering, the interaction of ATP with the actin-subfragment in the presence of  $Mg^{2+}$  does not show a lasting effect on the scattering of unpolarized light or on the polarization ratio which can be observed with actomyosin. The unique changes of superprecipitation are apparently brought about by the characteristic ability of light meromyosin to form a hydrated gel. Without the formation of gel, the actin-myosin complex may be regarded as a simple dissociation-association system.



## DISCUSSION

The work presented in this thesis has its immediate origin in the observations by the research group of H. Weber that  $\text{Mg}^{2+}$  is necessary for the actin-myosin system to convert the energy stored in ATP into mechanical work. As an approach to the problem of how  $\text{Mg}^{2+}$  acts in the contractile system, a comparative analysis of the effects of  $\text{Mg}^{2+}$  and  $\text{Ca}^{2+}$  on the reactions among actin, myosin, and ATP or ITP has been performed.

The question posed was whether the binding of divalent cations with myosin causes the change in enzymatic characteristics of this protein or whether the formation of cation-substrate complexes is responsible for changes in the rate of pyrophosphate bond splitting. The range of  $\text{Mg}^{2+}$  concentration in which the rate of ATP hydrolysis changes appears to indicate that a binding of  $\text{Mg}^{2+}$  to myosin modifies the enzymatic property of this protein. The measurement of  $\text{Mg}^{2+}$ -myosin binding performed using an ultrafiltration technique suggests that one atomic equivalent of  $\text{Mg}^{2+}$  binds with a mole of myosin with an affinity constant having a value of the order of  $10^6 \text{ M}^{-1}$ , which agrees with the kinetic data. With  $\text{Ca}^{2+}$  as a modifier of myosin ATPase, an analysis of the rate of ATP hydrolysis as a function of the  $\text{Ca}^{2+}$  concentration also suggests a binding reaction involving  $\text{Ca}^{2+}$ , which seems to coincide with the formation of  $\text{CaATP}^{2-}$ . Observations on the binding of  $^{45}\text{Ca}^{2+}$  to myosin illustrate that there are at least two types of  $\text{Ca}^{2+}$  binding to myosin; one is similar to the  $\text{Mg}^{2+}$  binding in the number of ions bound and in affinity, and the other has an affinity constant of the same order as that found in the  $\text{CaATP}^{2-}$ .



formation. The latter binding reaction was reported by Luchi and Kritcher (1967) as occurring at 8 binding sites per mole of myosin with an affinity constant estimated as  $1.8 \times 10^4 \text{ M}^{-1}$ . In this regard there are several reports (Nanninga, 1957; Blum and Felauer, 1959; Lowenstein, 1960; Tetas and Lowenstein, 1963) which support the idea that  $\text{CaATP}^{2-}$  is hydrolysed more rapidly by myosin than other ionic species of ATP. On the other hand, the weak binding of  $\text{Ca}^{2+}$  to myosin cannot be ruled out as the cause of the  $\text{Ca}^{2+}$  activation of myosin ATPase (Nanninga, 1959). It seems reasonable, therefore, to state that both  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$  can bind tightly to myosin, although only the binding of  $\text{Mg}^{2+}$  shows a direct influence on myosin ATPase.

There is, however, some evidence that the  $\text{Ca}^{2+}$  bound tightly to myosin affects its enzymatic activity indirectly. As shown in the experiments with ITP or pMB-modified myosin, the  $\text{Ca}^{2+}$  activation of the enzymatic activity is nullified by the addition of small amounts of  $\text{Mg}^{2+}$ , which apparently is the result of  $\text{Mg}^{2+}$  replacing  $\text{Ca}^{2+}$  at the tight binding site.

Concerning changes in the chemical nature of ATP caused by a bound cation,  $\text{MgATP}^{2-}$  does not seem to differ from  $\text{HATP}^{3-}$  and  $\text{ATP}^{4-}$  in the capacity to act as a substrate for ATPase. For instance, the maximum  $\text{Mg}^{2+}$  effect can be attained by adding 0.01 mM  $\text{Mg}^{2+}$  to a reaction mixture containing 0.1 mM ATP (Fig. 15). Under this condition less than 10% of total ATP is complexed. Further additions of  $\text{Mg}^{2+}$  increase  $\text{MgATP}^{2-}$  without affecting the kinetic parameters of myosin ATPase. However, the  $\text{Ca}^{2+}$  bound to ATP may affect the hydrolytic process as discussed above. In this connection, Lymn and Taylor (1970)



pointed out the possibility that  $\text{Ca}^{2+}$  accelerates the rate at which the product is released from the active site in myosin. The experimental data obtained in this work and by other researchers have not made clear, however, whether the  $\text{Ca}^{2+}$  binding to myosin or the  $\text{CaATP}^{2-}$  formation is responsible for the  $\text{Ca}^{2+}$  activation of myosin NTPase.

The relationship between the actomyosin ATPase activity and the concentration of  $\text{Ca}^{2+}$  or  $\text{Mg}^{2+}$  was shown to be remarkably similar to that observed for myosin ATPase, except that  $\text{Mg}^{2+}$  acts as an activator for actomyosin ATPase. This may seem to indicate that myosin reacts with these divalent cations without being affected by the bound actin. It is probable however, that F-actin contains certain amounts of  $\text{Mg}^{2+}$ , since it is usually polymerized in the presence of  $\text{Mg}^{2+}$ , and it may bind with the added cation, as the affinity of  $\text{Mg}^{2+}$  or  $\text{Ca}^{2+}$  to G-actin has been reported by Maruyama and Gergely (1962), to be of the same order of magnitude as that to myosin.

If the assay of actomyosin ATPase is performed in the absence of EDTA, a limited level of activity is observable even without added divalent cations. Upon addition of EDTA, ATP hydrolysis becomes undetectable. Although it is not clear whether or not EDTA chelates the divalent cation bound to actin, the fact is that at a KCl concentration below 0.1 M, actomyosin requires divalent cations to catalyse ATP hydrolysis, whereas monovalent cations alone can activate myosin ATPase.

Superprecipitation apparently involves several steps before reaching the final stage (Nihei and Yamamoto, 1969). A detection method for the physical changes in this process may follow any one or a combination of those steps. Although turbidity changes of actomyosin





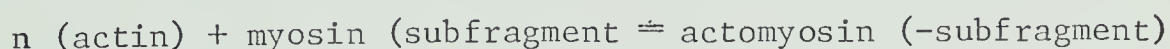
suspensions have often been used to detect aggregate formation, the rate and extent of superprecipitation so measured depend on the optical arrangement of the measuring device. The method used in this work is the one which gives the turbidity change following the first order kinetics at actomyosin concentrations lower than 0.05 mg/ml. The extent of superprecipitation with different ATP concentrations is constant at a fixed concentration of actomyosin. If the concentration of ATP is much reduced, the turbidity change ceases at the time of ATP exhaustion. According to a previous study by Nihei and Yamamoto (1969), the rate measured by the method used represents that of actomyosin gel dehydration. The experiments showed that both  $\text{Mg}^{2+}$  and  $\text{Ca}^{2+}$  activate superprecipitation maximally at 3-4 mM. The rate of ATP hydrolysis in the presence of 4 mM  $\text{Ca}^{2+}$  is 0.7-0.8  $\mu\text{mole}/\text{min}.\text{mg}$  protein, accompanied by the turbidity change at a rate giving a  $t_{1/2}$  of about 8 seconds, whereas with 4 mM  $\text{Mg}^{2+}$ , the rate becomes 0.4-0.5  $\mu\text{moles}/\text{min}.\text{mg}$ , and the turbidity changes at a rate indicating a  $t_{1/2}$  of about one second. It may be restated that if actomyosin is to undergo superprecipitation in the presence of a sufficient amount of  $\text{Ca}^{2+}$ , it needs about 60 moles of ATP hydrolysis to convert one mole of myosin into the superprecipitated state, whereas in case  $\text{Mg}^{2+}$  is the activator, actomyosin requires only 5 moles of ATP to be split per mole of myosin in completing the change. These observations suggest that the actin-myosin system is better adapted to  $\text{Mg}^{2+}$  in the process of coupling ATP hydrolysis to mechanical work.

It was thought then that if one could ascertain the quantitative relationship between actin and myosin in the formation of



actomyosin, it might be of value in understanding the nature of superprecipitation. In carrying out the experiments for this purpose, it has become apparent that since myosin is a large molecule with two active centers, a simpler system than actomyosin can be analysed on the basis of an uncomplicated chemical equilibrium. For this reason, attempts have been made to obtain homogeneous preparations of an active subfragment (HMM-S1) from myosin digested by papain. The subfragment prepared by the technique described in the Results appeared to be reasonably homogeneous as observed in the ultracentrifuge experiments, except that it tends to form aggregates unless it is in a solution containing  $\beta$ -mercaptoethanol. In the presence of 0.02 M of this reagent, the subfragment showed a higher ATPase activity (0.21  $\mu$ mole Pi/min.mg) in a reaction mixture containing 1.0mM ATP, 1.0mM  $\text{MgCl}_2$ , 0.05M tris-Cl (pH 7.4), and 1.0mM D.T.T. at  $25^\circ$  than in the absence of  $\beta$ -mercaptoethanol (0.11  $\mu$ mole Pi/min.mg). It may be that this reagent blocks the sulfhydryl groups of the subfragment by forming disulfide bonds, in which case the ATPase is increased as in the case of the pMB-treated myosin.

The analysis of the  $\text{Mg}^{2+}$  activated ATPase as a function of the ratio of actin to myosin or its subfragment is based on the assumption that in the presence of ATP and  $\text{Mg}^{2+}$ , actin and myosin or its active subfragment are at equilibrium with their complex as can be expressed by the scheme:



where  $n$  is an integer, and actin denotes a monomer incorporated in F-actin. If myosin, its subfragment, actomyosin, and acto-subfragment



possess distinct ATPase characteristics, in particular the rate constants of the slowest step, and if the total concentrations of actin and myosin or its subfragment are known, the equilibrium constant of the above reaction can be determined. The rate constant of myosin ATPase can be experimentally measured, whereas that of actomyosin (-subfragment) ATPase cannot be determined unequivocally, since it is difficult to secure the condition under which the amount of free myosin (or subfragment) becomes negligibly small. Hence assumed values of the rate constant for actomyosin (-subfragment) ATPase were used in the calculation of the relationship between the ATPase activity and the ratio of actin to myosin or subfragment. As described in the Results, both myosin and its subfragment appear to bind with F-actin at the molar ratio of one to one where monomer actin is taken as the molecular unit. Previously, Young (1967b) and Rizzino et al. (1970) reported a mole per mole association of actin to heavy meromyosin, although Szentkiralyi and Oplatka (1969) observed the second actin binding to heavy meromyosin at high concentrations of actin. It is generally agreed among several workers (Eisenberg et al., 1968; Szentkiralyi and Oplatka, 1969; Rizzino et al., 1970; see also Table 15) that the affinity of actin to heavy meromyosin or to active subfragment (HMM-S1) is much weaker than to myosin.

There is little doubt that at least two active centers exist in a molecule of myosin. It is not unreasonable to assume that these centers influence each other in their reactions with other molecules. The observations that a binding reaction of either  $\text{Ca}^{2+}$  or  $\text{Mg}^{2+}$  to myosin with relatively high affinity occurs at a site per



mole, suggest the influence of one cation on two active centers. There has been a discrepancy in the values of the Michaelis constant,  $K_m$ , for the myosin ATPase obtained in a high range of ATP concentration ( $K_m \approx 10^{-4}M$ ) and in low range ( $K_m \approx 10^{-7}M$ ). In studies of the proton release during the transient phase of myosin ATPase two reaction rates depending on the substrate concentration were recorded (Finlayson and Taylor, 1969). These may also mean that some interactions exist between the active centers of myosin (Lymn and Taylor, 1970). In the absence of evidence that the myosin subfragment is functionally heterogeneous in its binding with actin, the mole per mole binding between actin and myosin could be explained in terms of interactions between the two active centers in myosin. It should be pointed out that there could be two types of interactions; one is the competition between two active centers for one actin monomer and the other is the co-operation of the centers to attract actin. To determine which type of interaction is more feasible, one may consider the affinity of myosin to actin, which is stronger than that of the myosin subfragment. This phenomenon, however, is in accord with either type of the active center interaction, since the competition between two in the same molecule does not necessarily reduce the attractive force applied to the whole molecule.

It may be that the experimental conditions used in this study are far from physiological reality, under which myosin is unable to react with actin as it does in vivo. In fact, the ATPase activity of actomyosin as a function of the KCl concentration gives a bell-shaped curve (Fig. 21). In living muscle, ionic strength is approximately





0.15, and the concentration of ATP is kept constant at about 4-5mM. Under physiological conditions, the active centers of myosin and actin could be at equilibria more dynamic than the simple equilibrium assumed in analyzing the data.

In closing, the following conclusions can be stated: the binding reactions of  $\text{Ca}^{2+}$  to myosin are quantitatively similar to that of  $\text{Mg}^{2+}$ , though the effects of these cations on myosin ATPase appear qualitatively different. In the actomyosin system,  $\text{Mg}^{2+}$  as an essential factor for superprecipitation can be replaced by  $\text{Ca}^{2+}$ . Considering the greatly reduced efficiency of  $\text{Ca}^{2+}$  in this role, the interaction of actin and myosin as the basic reaction of muscular contraction seems to possess a high specificity, if not absolute, in its requirement for  $\text{Mg}^{2+}$ . In addition, the small quantity of  $\text{Ca}^{2+}$  relative to  $\text{Mg}^{2+}$  present in living muscle makes it nearly impossible for  $\text{Ca}^{2+}$  to act directly on the actin-myosin system in vivo. Secondly, the interaction of actin and myosin as observed in the dependence of  $\text{Mg}^{2+}$ -ATPase on the ratio of actin to myosin shows that only one actin monomer can bind to a mole of myosin. The binding of myosin subfragment (HMM-S1) to actin also indicates the same extent, i.e., a mole per mole binding. In view of the evidence currently available, the interaction between two active centers in a molecule of myosin appears to have become an important factor in understanding the mechanism of actin-myosin interaction.



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